

congo red DAP plates for determination of viable bacterial counts.

For visual examination of fixed and stained chamber slides, 1×10^5 BHK cells were plated in Nunc chamber slides and infected with 15D and 15D(pCMVB) as described above. At the appropriate times, chamber slides were extensively washed, fixed and stained with a Leukostain set (Fisher). At least 450 cells were visually examined by light microscopy for data analysis. An Instat statistical program (Graphpad, San Diego, CA) was used to calculate means and standard deviations.

EXAMPLE 3

Expression of DNA delivered to cells by strain 15D

Bacteria were grown as described in Example 1 except that the bacterial suspensions were concentrated 10-fold and 2 mls were added to each flask. In this assay, 50 $\mu\text{g/ml}$ of DAP was added to bacterial suspensions prior to their addition to flasks of semi-confluent BHK cells. Bacteria were added at a ratio of approximately 100:1. At the indicated time points, BHK cells were removed by trypsinization and washed in PBS. A portion of the cell suspension was lysed with a 0.2% Triton-X-100 solution and plated on TSA congo red DAP plates for determination of viable bacterial counts. The remainder of the cells were assayed for β -galactosidase activity. β -galactosidase activity was measured in the remaining cell extract by a standard biochemical assay that uses the conversion of o-nitrophenyl- β -D-galactoside (ONPG) to galactose and the chromophore o-nitrophenol to quantitatively detect activity spectrophotometrically (Nolan et al. in Methods in Molecular Biology, E. J. Murray and J. M. Walker, Eds. (Humana Press Inc., Clifton, N. J., 1991) Vol. 7: 217-235). Units of β -galactosidase = $380 \times \text{OD}_{420} / \text{Time (minutes)}$. Total protein concentrations of cellular extracts were determined via a BCA* protein assay kit (Pierce). Results are shown in Figure 2a and 2b.

Initially $1-3 \times 10^7$ viable bacteria of each strain were

recovered from monolayers of BHK cells with no detectable β -galactosidase activity in cell extracts. Measurements of β -galactosidase activity in bacterial extracts equivalent to the total number of bacteria added were negative. After 4 hours, a 1 log to 1.5 logs loss in viable bacteria occurred with no detectable β -galactosidase activity. An additional log to 1.5 logs loss of viable bacteria was observed at both the 24 and 48 hour assay points. At both times, increasing units of β -galactosidase activity were readily detectable in cell extracts from BHK cells infected with 15D(pCMV β). β -galactosidase activity detected at these last assay points was not due to expression from within the bacteria because no activity was detected at the first two assay points, yet a high level of viable bacteria were present. In addition, a noninvasive isolate of 15D(pCMV β) (i.e., IpaB and IpaC immunoblot negative) was tested for the ability to deliver plasmid DNA. No β -galactosidase activity was detected at the 24 hour assay point.

This finding reinforces the hypothesis that to deliver DNA the bacteria must be capable of entering the mammalian cell and breaking out of the phagocytic vacuole, which most likely occurs during the first 4 hours of this assay. By the 24 and 48 hour assay points, sufficient time had passed for death of the bacterium and release of the plasmid DNA into the cell cytoplasm. This is followed by transcription and translation of the encoded reporter gene. Extracellular lysis of bacteria leading to the release of plasmid DNA with subsequent uptake by eukaryotic cells cannot account for these findings since the noninvasive isolate was unable to induce β -galactosidase activity.

EXAMPLE 4

Strain 15D as a DNA delivery vehicle

To verify the delivery of pCMV β DNA to BHK cells, infected monolayers were immunostained to visually detect intracellular β -galactosidase expression within individual

cells. As described in Example 1, 3 wells of a 4-well chamber slide of BHK cell monolayers infected with either 15D or 15D(pCMVB) were immunostained to detect β -galactosidase expression (Sander et al. *J. immunol. Methods* (1993) 166:201).

5 At each assay point, monolayers were fixed in phosphate-buffered 4% paraformaldehyde for 5 min. and subsequently blocked with 3% goat serum (Gibco-BRL) in HBSS for 30 min. BHK cells were then permeabilized for 1 min. with HBSS containing 0.1% saponin (Sigma) solution. Monoclonal
10 anti- β -galactosidase (Sigma) was diluted 1:2000 in 0.1% saponin/HBSS and applied for 30 min. at 37°C in a humidified chamber. Secondary anti-mouse IgG (Fc specific) FITC conjugated (Sigma) was diluted 1:32 and applied for 30 min. at room temperature. Between each step chamber slides were
15 washed extensively with 0.1% saponin/HBSS solution. A final wash step of HBSS alone was used to close permeabilized cells. Fluorescent images were visualized with either a Nikon microphot with Epi-fluorescence attachment or an Olympus-VAN04-S with fluorescence attachment. Results are
20 shown in Figure 3.

No apparent intracellular immunostaining was observed in monolayers infected with either strain at the 30 minute assay point (Figure 3A, B). Only slight intracellular immunostaining was detected at the 4 hour assay point in monolayers infected
25 with 15D(pCMVB) (Figure 3C, D). At the 24 and 48 hour assay points, several cells per field of monolayers infected with 15D(pCMVB) were positively stained (Figure 3E, F). Staining throughout the cell cytoplasm indicated that the plasmid DNA had been released from the bacterium into the cell cytoplasm
30 for further processing (i.e., transcription and translation) by the mammalian cell. Positively staining cells also appeared to be rounded, possibly due to the presence of an extensive amount of β -galactosidase protein. Approximately 1-2% of 5000 cells were stained positive for β -galactosidase
35 expression at the 24 hour assay point as determined by

fluorescence activated cell sorter (FACS) analysis (Nolan et al., supra). Visual examination of Leukostat stained chamber slides of 15D(pCMV β) infected BHK cells demonstrated that 28% of the cells contained 1 to 5 intact bacterial cells with 1.7% containing 5 bacteria (Table 2). Four hours after gentamicin treatment 26% of the cells contained visually intact bacteria with less than 1% of the cells containing 4 bacteria.

Therefore, invasion with between 1-5 bacteria was required for foreign gene expression. Since pCMV β is a 7164 base pair plasmid of medium to high copy number with approximately 500 copies per bacterial cell, each bacterium is estimated to contain about 3.93×10^{-9} mg of DNA. Intracytoplasmic delivery of approximately $4-20 \times 10^{-9}$ mg of DNA by *Shigella* is sufficient for expression of β -galactosidase.

Table 2. Visual examination of infected BHK cells.

Strain	Time	% Infected	Bacteria per BHK mean (SD)	Total number of BHK cells containing:						
				Number of Bacteria:						
				1	2	3	4	5	6	Total:
15D	30'	39.3	1.84 (1.2)	96	47	14	14	3	3	177
	4 h	35.8	1.68 (0.94)	106	36	13	5	0	1	161
	24 h	3.7	1	-	-	-	-	-	-	-
	48 h	2.2	1	-	-	-	-	-	-	-
pCMV β	30'	28	1.35 (0.72)	76	29	7	5	2	0	119
	4h	25.95	1.4 (0.74)	95	16	4	1	0	0	116
	24 h	3.3	1	-	-	-	-	-	-	-
	48 h	3.8	1	-	-	-	-	-	-	-

Percentage of BHK cells infected and number of bacteria per infected BHK cell. Chamber slides and bacteria were prepared as described in Table 1. Data are presented as the mean percentage of infected BHK cells and mean \pm standard deviation (SD) of bacteria per infected BHK cell.

EXAMPLE 5

Gene delivery by *Shigella* to different cell types

Shigella species invade many different types of cells. To demonstrate that gene delivery was not restricted to BHK cells, P815 cells were infected with 15D(pCMV β). Bacteria used to infect P815 cells were grown as described in Example

1. After the addition of the bacteria with DAP to the non-adherent P815 cells cultured in 6-well plates, the plate was spun at 500 X g for 5 minutes. Bacteria and P815 cells were allowed to interact for 90 minutes. The cells were then extensively washed with DMEM and resuspended in DMEM containing 100 µg/ml gentamicin for a one hour incubation at 37°C, 5% CO₂. The cells were again extensively washed and resuspended in DMEM containing 20 µg/ml gentamicin for overnight culture at 37°C, 5% CO₂. β-galactosidase activity and protein concentrations were determined at 24 hours as described (Nolan et al., supra).

As shown in Table 3, 10 fold higher levels of β-galactosidase were expressed compared to background control at 24 hours. P815 cells, which express H-2^d class I MHC molecules, have been successfully infected with 15D(pCMVB) and experiments are currently underway to determine if these cells can present *Shigella* delivered DNA encoded foreign antigens in the context of class I.

Table 3. β-galactosidase activity in P815 cells after infection with 15D(pCMVB).

Source:	Units of β-galactosidase/mg protein:
P815 cells	3.04
P815 cells + 15D	5.62
P815 cells + 15D(pCMVB)	56.25

EXAMPLE 6

15D provides protection against infection by shigella in vivo

Experiments in a guinea pig keratoconjunctivitis challenge model demonstrate 100% protection from subsequent *Shigella* infection three weeks following a two dose immunization regime. Animals were immunized with 1-4 x 10⁸ colony forming units per eye on days 0 and 15. Challenge occurred 3 weeks after final immunization. Animals were challenged with 3.8 x 10⁸ virulent 2457T.

Table 4. Guinea Pig Challenge Summary

EXP.	No. of eyes with rating of:					Protection:		
	0	1	2	3	4	Full	Partial	Combined %
5 A								
1x dose	2	2	0	0	0	50	50	100
5x dose	1	1	0	0	0	50	50	100
Control	0	0	0	0	4			
After immunizations on days 0 and 14, animals were challenged 3 weeks later with 2.5×10^8 virulent 2457T.								
10 B								
1x dose	2	2	0	0	0	50	50	100
5x dose	2	0	0	0	0	100	0	100
Control	0	0	0	0	10			
After immunization on days 0 and 14, animals were challenged 3 weeks later with 5×10^8 virulent 2457T.								
15 *Animals above were immunized with between $2.5-3 \times 10^8$ colony forming units per eye with strain 15D on days 0 and 14.								
C								
Strain:								
15D	2	6	0	0	0	25	75	100
20 pCMVB 1	7	0	0	0	0	13	87	100
Heat-killed								
pCMVB 0	4	4	0	0	0	50	50	
Controls 0	0	0	6	2		0	0	0
25 pCMVB: 15D carrying a commercially available eukaryotic expression plasmid.								
Heat-killed: heat to 56°C for 30 minutes.								

Eyes from animals in experiment C were also stained for β -galactosidase activity. Eyes from animals inoculated with 15D(pCMVB) and 15D(pCMVB) heat-killed showed staining. Less staining was detected in heat-killed 15D(pCMVB) inoculated animals. These results demonstrate that this highly attenuated strain, which is capable of DNA delivery, functions well *in vivo* in the guinea pig keratoconjunctivitis model, and provides protection against challenge with *Shigella*, even when the bacteria is inactivated.

EXAMPLE 7

Guinea Pig Proliferation Assay

The purpose of this experiment was to determine the immune responsiveness of animals at the time of challenge as well as during the recovery period.

The spleens or cervical nodes of two animals were pooled for testing. Two challenged animals from each group were sacrificed 3 and 4 weeks post challenge for testing. Proliferative responses were tested on animals being analyzed for protection. Pre-challenge-animals were vaccinated as described and organs tested at the time other animals were

being challenged.

Spleens and cervical nodes were processed to a single cell suspension and plated in 96 well plates at a concentration of $1-2 \times 10^5$ cells per well in 100 μ l. Ten μ l of
5 each stimulus was added to the appropriate wells. After three days in culture, the amount of proliferation that had taken place was measured using a non-radioactive kit. Responses are presented in Table 5 below.

Table 5: Stimulation Index

		<u>Spleen</u>			<u>Cervical Nodes</u>		
		ConA	LPS	H.K.	ConA	LPS	H.K.
prechallenge							
5	15D	3.9	1.6	1.85	0.42	NP.	2.3
	15D(pCMVB)	2.2	1.2	0.9	2.46	1.55	3.2
	Heat-killed 15D(pCMVB)	1.15	0.7	0.675	1.15	3.55	2.8
3 weeks post-challenge							
10	15D	0.78	4.25	2.4	2.36	N.P.	1.18
	15D(pCMVB)	0.77	4.25	1.5	0.56	N.P.	0.59
	Heat-killed 15D(pCMVB)	0.87	N.P.	N.P.	0.54	8.25	1.9
4 weeks post-challenge							
15	15D	2.05	N.P.	(0.039)*	0.79	N.P.	0.23
	15D(pCMVB)	1.8	(0.036)*	N.P.	0.30	0.69	0.26
	Heat-killed 15D(pCMVB)	0.89	(0.130)*	(0.105)*	0.68	0.31	0.38
20	Challenged Naive	2.08	(0.180)*	(0.091)*	0.52	1.69	0.56

N.P. - no proliferation detected

* - naive animal showed no detectable response: therefore, actual O.D. values are presented.

ConA- concanavalin A 5µg/ml

LPS- commercial preparation from *E.coli* 250pg/ml

H.K.- heat-killed *Shigella flexneri* 2a strain 2457T 5µg/ml

All responses were averaged (i.e., 3-4 wells) and the average background response subtracted to determine the O.D. 490 values. Stimulation index was calculated by dividing the average experimental O.D. value by that of the naive control.

These results give insight into the immune responses (T cell and B cell involvement as measured by mitogenic responses, and specific responses to heat-killed antigen) to this highly attenuated strain at the time of challenge and during the weeks post challenge. Proliferation to B-galactosidase protein was not detected. Due to the normal immunological characteristics of the eye, this result was

expected (Rocha and Baines *Critical Rev. Immun.* (1992) 12:81-100).

EXAMPLE 8

Mouse Intranasal Challenge Proliferation

5 The purpose of this experiment was to measure in an alternative model (i.e. murine intranasal) the ability of 15D to deliver DNA *in vivo*. In addition, immune responses to the carrier were also determined.

10 Groups of five mice each were inoculated twice intranasally 4 weeks apart. For each strain or treatment, three different doses were also given. Amounts are indicated below. One treatment group consisted of mice given 15D(pCMVB) with 50 µg/ml of DAP added to the culture prior to inoculation. Four weeks after the second inoculation, spleens
15 were removed, processed to a single cell suspension and plated in 96 well plates at 2×10^5 cells per well in 100 µl. Ten µl of the stimuli were added to the appropriate wells. Plates were incubated for three days, and the amount of proliferation that had taken place was measured using a non-radioactive kit.
20 Values were averaged and the background subtracted to determine the O.D. 490 value. Stimulation index for ConA, *E.coli* LPS and heat killed 2457T was calculated by dividing the average experimental O.D. value by that of the naive control. Results are shown in Table 6 below. Stimulation
25 Index for b-gal is experimental (pCMVB) O.D. value divided by that of 15D.

Tabl 6: Stimulation Index

Index=pCMVB/15D		Stimulation Index=Exp/Control			Stimulation	
		ConA 5 µg/ml	<i>E.coli</i> LPS 250 pg/ml	Heat-killed 2457T5 µg/ml	β-gal protein [^] 0.25 µg/ml	b-gal protein [^] 2.5 µg/ml
5	15D (high)	1.16	0.71	0.93	----	----
	(middle)	1.34	0.68	0.73	----	----
	(low)	1.10	0.52	0.84	----	----
10	15D(pCMVB) (high)	1.22	0.57	1.34	2.37	2.09
	(middle)	1.12	0.77	1.49	2.09	2.39
	(low)	1.15	0.61	1.17	0.66	0.7
15	15D(pCMVB+ DAP (high)	0.85	1.29	1.27	3.12	3.6
	(middle)	1.16	0.50	0.82	0.62	0.90
	(low)	1.19	0.34	0.69	0.20	0.60

Approximate dose for both inoculations:

15D- 3 X 10⁶, 1 X 10⁶ and 3 X 10⁵

15D(pCMVB) with or without DAP- 1 X 10⁶, 5 X 10⁵, 1 X 10⁵

[^] polymyxin B was added to the b-gal protein to chelate any contaminating LPS.

These results indicate that in this model, 15D can successfully deliver pCMVB DNA. At higher inoculating doses, mice that have been inoculated with 15D(pCMVB) with or without the addition of DAP are capable of proliferating in response to b-gal protein. In addition, there was no significant proliferative responses to the carrier at the doses given.

EXAMPLE 9

Mouse Intranasal Response II

Lymphoproliferative and antibody responses directed against the plasmid expressed β-galactosidase were measured after bacterial delivery of plasmid DNA to the nasal tissue of mice. Two intranasal inoculations were administered on days 0 and 28. Four weeks after the last inoculation, splenocytes from mice receiving 15D(pCMVB) showed lymphoproliferative responses directed against β-galactosidase. Eight to 10 week-old female BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were sedated by intramuscular injection of a mixture of 0.3 mg xylazine hydrochloride (Rompun; Mobay Corp., Shawnee, KA) and 1.0 mg of ketamine hydrochloride (Ketaset; Aveco Company, Fort Dodge, IA) in 50 ml of saline. A concentrated bacterial suspension (15 ml) was dropped onto the external nares of each mouse. Mice in groups of 5 to 10 were administered either 10⁶

or 10^7 viable bacteria on day 0 and 4 weeks. Some groups of mice received inocula of 15D(pCMVB) supplemented with 50 $\mu\text{g/ml}$ of DAP. Blood for serum analysis was collected 4 weeks after the last inoculation. At that time, spleens were also removed for *in vitro* determination of lymphoproliferative responses induced by ConA, *E.coli* LPS, heat-killed 2457T, and purified β -galactosidase (Sigma, St. Louis, MO). Splenocytes ($1 \times 10^5/\text{well}$) were cultured in the presence of 5 $\mu\text{g/ml}$ ConA, 2.5 $\mu\text{g/ml}$ *E.coli* LPS, 5 $\mu\text{g/ml}$ heat-killed 2457T, and 2.5 $\mu\text{g/ml}$ β -galactosidase with 10 $\mu\text{g/ml}$ polymixin B (Burroughs Wellcome, Research Triangle Park, NC) for 3 days. Levels of proliferation were determined using a Cell Titer 96TM AQueous non-radioactive cell proliferation kit (Promega, Madison, WI). Reported OD490 values were calculated by subtracting the mean value of unstimulated cells from the mean value of stimulated cells.

Results indicate that mice inoculated with 15D(pCMVB) with or without the addition of DAP are capable of proliferating in response to β -galactosidase, up to five-fold higher than controls (Figure 4D).

EXAMPLE 10

Antibody responses to β -galactosidase of intranasally inoculated mice

Sera from groups of mice inoculated with either 15D, 15D(pCMVB), or 15D(pCMVB) containing 50 $\mu\text{g/ml}$ of DAP were tested for reactivity to β -galactosidase. One microgram of purified β -galactosidase was electrophoresed on 7.5% SDS-polyacrylamide gels. After electrophoresis, gels were electroblotted to nitrocellulose. Casein blocked blots were then sectioned before overnight exposure to pooled sera samples (diluted 1:50 in casein buffer). Bound antibody was detected with a 1:500 dilution of secondary rabbit anti-mouse Ig conjugated with alkaline phosphatase (BMB, Indianapolis, IN). Alkaline phosphatase activity was detected by substrates BCIP/NBT (Sigma). Immunoblot analysis revealed antibody responses specific for β -galactosidase in sera samples from

mice infected with 15D(pCMVB).

Sera samples were also analyzed by ELISA to determine antibody isotype and IgG subclass using standard methodology. Antibody specific for β -galactosidase was of the IgG isotype with IgG1, IgG2a, and IgG2b subclasses equally represented (Table 7), indicating involvement of both Th1 and Th2 cells.

Table 7: ELISA results

Animals inoculated with:		Anti- β -galactosidase Total IgG Titer:
	saline	0
10	15D 10^7	1:100
	15D 10^6	0
	15D(pCMVB) 10^7	1:12800
	15D(pCMVB) 10^6	1:800
	15D(pCMVB) + DAP 10^7	1:6400
15	15D(pCMVB) + DAP 10^6	0

IgG Subclass Typing

Animals inoculated with:		Anti- β -galactosidase:		
		IgG1	IgG2a	IgG2b
20	15D(pCMVB) 10^7	1:25600	1:25600	1:6400
	15D(pCMVB) 10^6	1:800	1:1600	1:1600
	15D(pCMVB) + DAP 10^7	1:3200	1:12800	1:3200

The results presented here represent the first evidence that attenuated bacteria can be used to deliver plasmid DNA to mucosal surfaces with subsequent stimulation of immune responses directed against the plasmid encoded foreign gene product. This approach to vaccine development should simplify production and delivery of DNA-based vaccines, while expanding the technology to allow stimulation of often desired mucosal immune responses.

We have discovered a novel method for delivering functional DNA inside cells. This method should not be

restricted to *Shigella*, since the invasion genes that *Shigella* utilizes can be inserted into other bacteria such as *E. coli* (Sansonetti et al. *Infect. Immun.* (1983) 39:1392). Likewise, other bacteria such as *Listeria* are able to invade cells and break out of the phagocytic vacuole into the cytoplasm (Portnoy and Jones, *Ann. N.Y. Acad. Sci.* (1994) 730:15). Although we have no formal proof that release from the phagocytic vacuole into the cell cytoplasm by the bacteria is essential for DNA delivery, preliminary experiments with *Salmonella typhimurium*, an organism that reaches the cytoplasm only with difficulty, suggests this organism is not an efficient DNA delivery vehicle.

Any bacterial vector DNA delivery system will need to strike a balance between cell invasion with its subsequent reactogenicity and efficiency of delivery. In the case of *Shigella*, the genes responsible for invasion also cause invasion and apoptosis of macrophages followed by inflammation (Zychlinsky et al. *Nature* (1992) 358:167). We constructed a *Shigella* strain that in the absence of DAP, is unable to survive inside the cell. Determination of the safety of this strain awaits human trials.

The bacterial DNA delivery system which we describe has several advantages for certain applications. Delivery of DNA encoded antigens to the mucosal immune system should permit mucosal immunization simultaneously with multiple antigens that can be directed for class I and/or II presentation, stimulation of Th1 or Th2 help, or secreted maintaining the proper folding and conformational epitopes for IgA and IgG antibody production. Diarrheal diseases such as rotavirus; sexually transmitted diseases such as human immunodeficiency virus, *Neisseria gonorrhoeae*, and human papilloma virus; and gastrointestinal diseases such as the ulcer causing *Helicobacter pylori* , to name a few, may be especially responsive to this approach. Suppression of autoimmunity through manipulation of gut immune tolerance mechanisms has been demonstrated (Sun et al. *Proc. Natl. Acad. Sci. U.S.A.*

(1994) 91: 10795), and should also be amenable to this approach.

Perhaps the greatest advantage of bacterial delivery of DNA for vaccination and potential gene therapy/replacement is the ease and acceptability of oral and other forms of mucosal delivery. Likewise, because no DNA purification is required for this type of DNA vaccination, which is really a live, attenuated bacterial vector, vaccines can be produced for the cost of fermentation, lyophilization and packaging.

Therefore, this type of vaccination may represent at least in part a solution to the cost and difficulty of current vaccines and those that are being developed.

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 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM compatible
 - (C) OPERATING SYSTEM: DOS/Windows 3.1
 - (D) SOFTWARE: WordPerfect 6.0
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1674 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

SEQUENCE DESCRIPTION: SEQ ID NO: 1

TCCATAATCA GGATCAATAA AACTGCTGCA GAAATGATTT	40
CATTCATAAC TCAAATTCCC TGATAATTGC CGCGGACTTT	80
CTGCGTGCTA ACAAAGCAGG ATAAGTCGCA TTAATCATGG	120
CTTCGCTATC ATTGATTAAT TTCACTTGCG ACTTTGGCTG	160
CTTTTTGTAT GGTGAAAGAT GTGCCAAGAG GAGACCGGCA	200
CATTTATACA GCACACATCT TTGCAGGAAA AAAACGCTTA	240
TGAAAAATGT TGGTTTTATC GGCTGGCGCG GTATGGTCGG	280
CTCCGTTCTC ATGCAACGCA TGGTTGAAGA GCGCGACTTC	320
GACGCCATTC GCCCTGTCTT CTTTCTACT TCTCAGCTTG	360
GCCAGGCTGC GCCGTCTTTT GGCAGAACCA CTGGCACACT	400
TCAGGATGCC TTTGATCTGG AGGCGCTAAA GGCCCTCGAT	440
ATCATTGTGA CCTGTCAGGG CGGCGATTAT ACCAACGAAA	480
TCTATCCAAA GCTTCGTGAA AGCGGATGGC AAGGTTACTG	520
GATTGACGCA GCATCGTCTC TGCATGATGAA AGATGACGCC	560
ATCATCATTC TTGACCCCGT CAATCAGGAC GTCATTACCG	600
ACGGATTAAA TAATGGCATC AGGACTTTTG TTGGCGGTAA	640
CTGTACCGTA AGCCTGATGT TGATGTCGTT GGGTGGTTTA	680
TTGCGCAATG ATCTTGTTGA TTGGGTGTCC GTTGCAACCT	720
ACCAGGCCGC TTCCGGCGGT GGTGCGCGAC ATATGCGTGA	760

GTTATTAACC CAGATGGGCC ATCTGTATGG CCATGTGGCA	800
GATGAACTCG CGACCCCGTC CTCTGCTATT CTCGATATCG	840
AACGCAAAGT CACAACCTTA ACCCGTAGCG GTGAGCTGCC	880
GGTGGATAAC TTTGGCGTGC CGCTGGCGGG TAGCCTGATT	920
CCGTGGATCG ACAAACAGCT CGATAACGGT CAGAGCCGCG	960
AAGAGTGGAA AGGGCAGGCG GAAACCAACA AGATCCTCAA	1000
CACATCTTCC GTAATTCCGG TAGATGGTTT ATGTGTGCGT	1040
GTCGGGGCAT TGCCTGCCA CAGCCAGGCA TTCACTATTA	1080
AATTGAAAAA AGATGTGTCT ATTCCGACCG TGAAGAAGT	1120
GCTGGCTGCG CACAATCCGT GGGCGAAAGT CGTTCCGAAC	1160
GATCGGGAAA TCACTATGCG TGAGCTAACC CCAGCTGCCG	1200
TTACCGGCAC GCTGACCACG CCGGTAGGCC GCCTGCGTAA	1240
GCTGAATATG GGACCAGAGT TCCTGTCAGC CTTTACCGTG	1280
GGCGACCAGC TGCTGTGGGG GGCCGCGGAG CCGCTGCGTC	1320
GGATGCTTCG TCAACTGGCG TAATCTTTAT TCATTAAATC	1360
TGGGGCGCGA TGCCGCCCTT GTTAGTGCGT AATACAGGAG	1400
TAAGCGCAGA TGTTTCATGA TTTACCGGGA GTTAAATAGA	1440
GCATTGGCTA TTCTTTAAGG GTGGCTGAAT ACATGAGTAT	1480
TCACAGCCTT ACCTGAAGTG AGGACGACGC AGAGAGGATG	1520
CACAGAGTGC TGCGCCGTTC AGGTCAAAAA AATGTCACAA	1560
CCAGAAGTCA AAAATCCAAT TGGATGGGGT GACACAATAA	1600
AACAGGAAGA CAAGCATGTC CGATCGTATC GATAGAGACG	1640
TGATTAACGC GCTAATTGCA GGCCATTTTG CGGA	1674

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(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1121 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: The *E. coli asd* gene coding for b-aspartic semialdehyde dehydrogenase identified in SEQ ID NO:1 was modified by deleting 553 bp from position 439 to 991.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

TCCATAATCA GGATCAATAA AACTGCTGCA GAAATGATTT	40
CATTCATAAC TCAAATTCCC TGATAATTGC CGCGGACTTT	80
CTGCGTGCTA ACAAAGCAGG ATAAGTCGCA TTA CTCATGG	120
CTTCGCTATC ATTGATTAAT TTCACTTGCG ACTTTGGCTG	160
CTTTTTGTAT GGTGAAAGAT GTGCCAAGAG GAGACCGGCA	200
CATTTATACA GCACACATCT TTGCAGGAAA AAAACGCTTA	240
TGAAAAATGT TGGTTTTATC GGCTGGCGCG GTATGGTCGG	280
CTCCGTTCTC ATGCAACGCA TGGTTGAAGA GCGCGACTTC	320
GACGCCATTC GCCCTGTCTT CTTTCTACT TCTCAGCTTG	360
GCCAGGCTGC GCCGTCTTTT GGCGGAACCA CTGGCACACT	400
TCAGGATGCC TTTGATCTGG AGGCGCTAAA GGCCCTCGGA	440
TCCTCAACAC ATCTTCCGTA ATTCCGGTAG ATGGTTTATG	480
TGTGCGTGTC GGGGCATTGC GCTGCCACAG CCAGGCATTC	520
ACTATTAAAT TGAAAAAAGA TGTGTCTATT CCGACCGTGG	560
AAGAACTGCT GGCTGCGCAC AATCCGTGGG CGAAAGTCGT	600
TCCGAACGAT CGGGAAATCA CTATGCGTGA GCTAACCCCA	640
GCTGCCGTTA CCGGCACGCT GACCACGCCG GTAGGCCGCC	680
TGCGTAAGCT GAATATGGGA CCAGAGTTCC TGTCAGCCTT	720

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TACCGTGGGC GACCAGCTGC TGTGGGGGGC CGCGGAGCCG	760
CTGCGTCGGA TGCTTCGTCA ACTGGCGTAA TCTTTATTCA	800
TTAAATCTGG GGC GCGATGC CGCCCCTGTT AGTGCGTAAT	840
ACAGGAGTAA GCGCAGATGT TTCATGATTT ACCGGGAGTT	880
AAATAGAGCA TTGGCTATTC TTAAAGGGTG GCTGAATACA	920
TGAGTATTCA CAGCCTTACC TGAAGTGAGG ACGACGCAGA	960
GAGGATGCAC AGAGTGCTGC GCCGTT CAGG TCAAAAAAAT	1000
GTCACAACCA GAAGTCAAAA ATCCAATTGG ATGGGGTGAC	1040
ACAATAAAAC AGGAAGACAA GCATGTCCGA TCGTATCGAT	1080
AGAGACGTGA TTAACGCGCT AATTGCAGGC CATTTTGCGG	1120
A	1121

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

AGATCTCCCTGATAATTGCCGC

22

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

AGATCTCGCTTACTCCTGTATTACGC

26

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(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5

CGAGGGCCTTTAGCGCCTCC

20

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6

GATCCTCAACACATCTTCCG

20

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7

GAGCTCCCCTGATAATTGCCGC

22

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(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8

GTCGACCGCTTACTCCTGTATTACGC

26

What is claimed is:

1. An attenuated *Shigella* strain wherein said *Shigella* is able to enter a cell and die once inside the cell.

5 2. An attenuated *Shigella* strain according to claim 1, wherein said strain is *S. flexneri*.

 3. The attenuated *Shigella* strain according to claim 2, wherein said strain is 15D given ATCC accession number ATCC
10 55710.

 4. A method for producing an attenuated *Shigella* strain, said method comprising inactivating an aspartate
b-semialdehyde dehydrogenase gene present in said *Shigella*.

15 5. A method for producing an attenuated *Shigella* strain according to claim 4 wherein said inactivation is by mutation.

 6. A method for producing an attenuated *Shigella* strain
20 according to claim 4 wherein said attenuated *Shigella* is able to enter a cell but dies once inside the cell.

 7. A vaccine for reducing in an individual disease symptoms caused by *Shigella*, said vaccine comprising:

- 25 (i) attenuated *Shigella*; and
 (ii) a pharmaceutically acceptable excipient.

 8. A vaccine for reducing in an individual disease symptoms according to claim 7, wherein said *Shigella* is *S.*
30 *flexneri*.

 9. A vaccine for reducing in an individual disease symptoms caused by *S. flexneri* according to claim 8, wherein said attenuated *S. flexneri* is 15D given ATCC accession number
35 ATCC 55710.

10. A vaccine for reducing in an individual disease symptoms caused by *Shigella* according to claim 7, wherein said attenuated *Shigella* is further inactivated.

11. A method for reducing in an individual disease symptoms caused by *Shigella* comprising administering to said individual attenuated *Shigella* in a pharmaceutically acceptable excipient, in an immunologically effective dose.

12. A method for reducing in an individual disease symptoms caused by *Shigella* according to claim 11, wherein said *Shigella* is *S. flexneri*.

13. A method for reducing in an individual disease symptoms caused by *Shigella* according to claim 11, wherein said attenuated *Shigella* is further inactivated.

14. A delivery vehicle for the delivery of DNA to a cell, said vehicle comprising attenuated *Shigella* wherein said DNA is introduced.

15. A delivery vehicle for the delivery of DNA to a cell according to claim 14, wherein said *Shigella* is *S. flexneri*.

16. A delivery vehicle for the delivery of DNA to a cell according to claim 14, wherein said cell is a cell of an intestinal mucosal epithelium cell.

17. A delivery vehicle for the delivery of DNA to a cell according to claim 14, wherein said *Shigella* is *S. flexneri*.

18. A delivery vehicle for the delivery of DNA to a cell according to claim 17, wherein said *S. flexneri* is 15D given ATCC accession number ATCC 55710.

19. A delivery vehicle for the delivery of DNA to a cell according to claim 14, wherein said attenuated *Shigella* is further inactivated.

5 20. A delivery vehicle for the delivery of an antigen to a cell comprising attenuated *Shigella* into which said antigen is introduced.

10 21. A delivery vehicle for the delivery of an antigen to a cell according to claim 20, wherein said *Shigella* is *S. flexneri*.

15 22. A delivery vehicle for the delivery of an antigen to a cell according to claim 21, wherein said *S. flexneri* is 15D.

20 23. A delivery vehicle for the delivery of an antigen to a cell according to claim 20, wherein said attenuated *Shigella* is further inactivated.

25 24. A method for oral immunization of an individual against *Shigella* comprising orally administering to said individual an immunologically effective amount of attenuated *Shigella* in a pharmaceutically acceptable excipient.

30 25. A method for oral immunization of an individual against *Shigella* according to claim 24, wherein said *Shigella* is *S. flexneri*.

35 26. A method for oral immunization of an individual against *Shigella* according to claim 25, wherein said *S. flexneri* is 15D.

 27. A method for oral immunization of an individual against *Shigella* according to claim 24, wherein said attenuated *Shigella* is further inactivated.

28. A method for delivering DNA to a cell, said method comprising:

(i) introducing said DNA into attenuated *Shigella*;

and

(ii) administering said *Shigella* to said cell.

29. A method for delivering DNA to a cell according to claim 28, wherein said *Shigella* is *S. flexneri*.

30. A method for delivering DNA to a cell according to claim 29, wherein said *S. flexneri* is 15D.

31. A method for delivering DNA to a cell according to claim 28, wherein said cell is a cell of a mucosal epithelium.

32. A method for delivering DNA to a cell according to claim 31, wherein said mucosal epithelium is intestinal mucosal epithelium.

33. A method for delivering DNA to a cell according to claim 28, wherein said attenuated *Shigella* is further inactivated.

34. A method for delivering an antigen to a cell comprising:

(i) introducing said antigen into an attenuated *Shigella*; and

(ii) administering said *Shigella* to said cell.

35. A method for delivering an antigen to a cell according to claim 34, wherein said *Shigella* is *S. flexneri*.

36. A method for delivering an antigen to a cell according to claim 35, wherein said *S. flexneri* is 15D given ATCC accession number ATCC 55710.

37. A method for delivering an antigen to a cell according to claim 34, wherein said cell is a cell of a mucosal epithelium.

5 38. A method for delivering an antigen to a cell according to claim 37, wherein said mucosal epithelium is intestinal mucosal epithelium.

10 39. A method for delivering an antigen to a cell according to claim 34, wherein said attenuated *Shigella* is further inactivated.

40. A method for detecting *Shigella* infection, said method comprising:

15 (i) coating a surface with attenuated *Shigella* or its components;

 (ii) contacting said coated surface with serum or tissue sample from an individual suspected of having said infection; and

20 (iii) detecting the presence or absence of the infection by detecting the presence or absence of a complex formed between said *Shigella* and immune response specific therefor present in said sample.

25 41. A diagnostic kit for the detection of *Shigella* infection, said kit comprising attenuated *Shigella*, and ancillary reagents suitable for use in detecting the presence of immune response to said *Shigella* in a sample.

30 42. A diagnostic kit for the detection of *Shigella* according to claim 41, wherein said *Shigella* is *S. flexneri*.

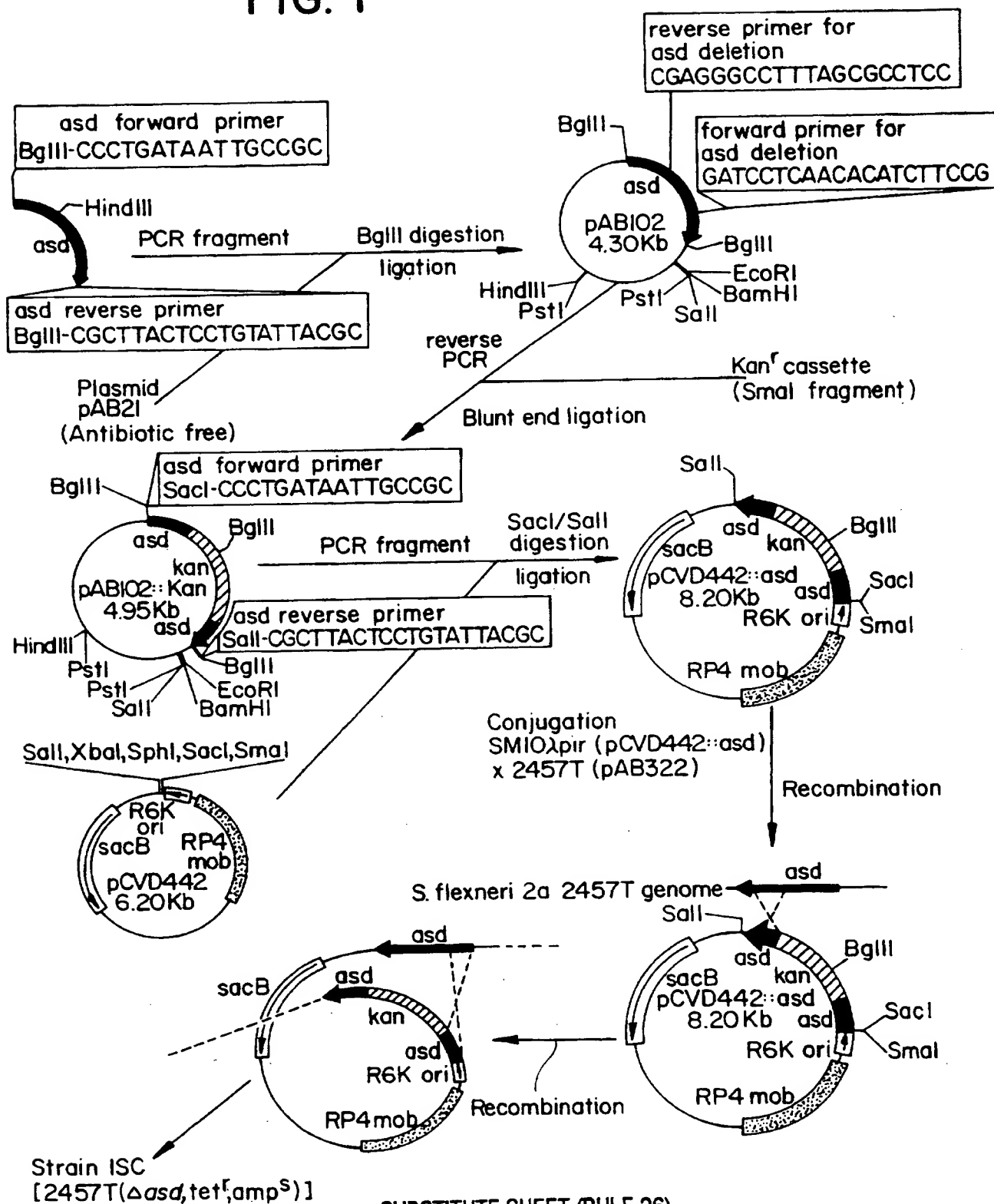
43. The diagnostic kit according to claim 42, wherein said *S. flexneri* is 15D given ATCC accession number ATCC
35 55710.

44. A method for the delivery of functional nucleic acids into a cell using bacteria comprising:

(i) introducing said nucleic acids into an attenuated bacteria; and

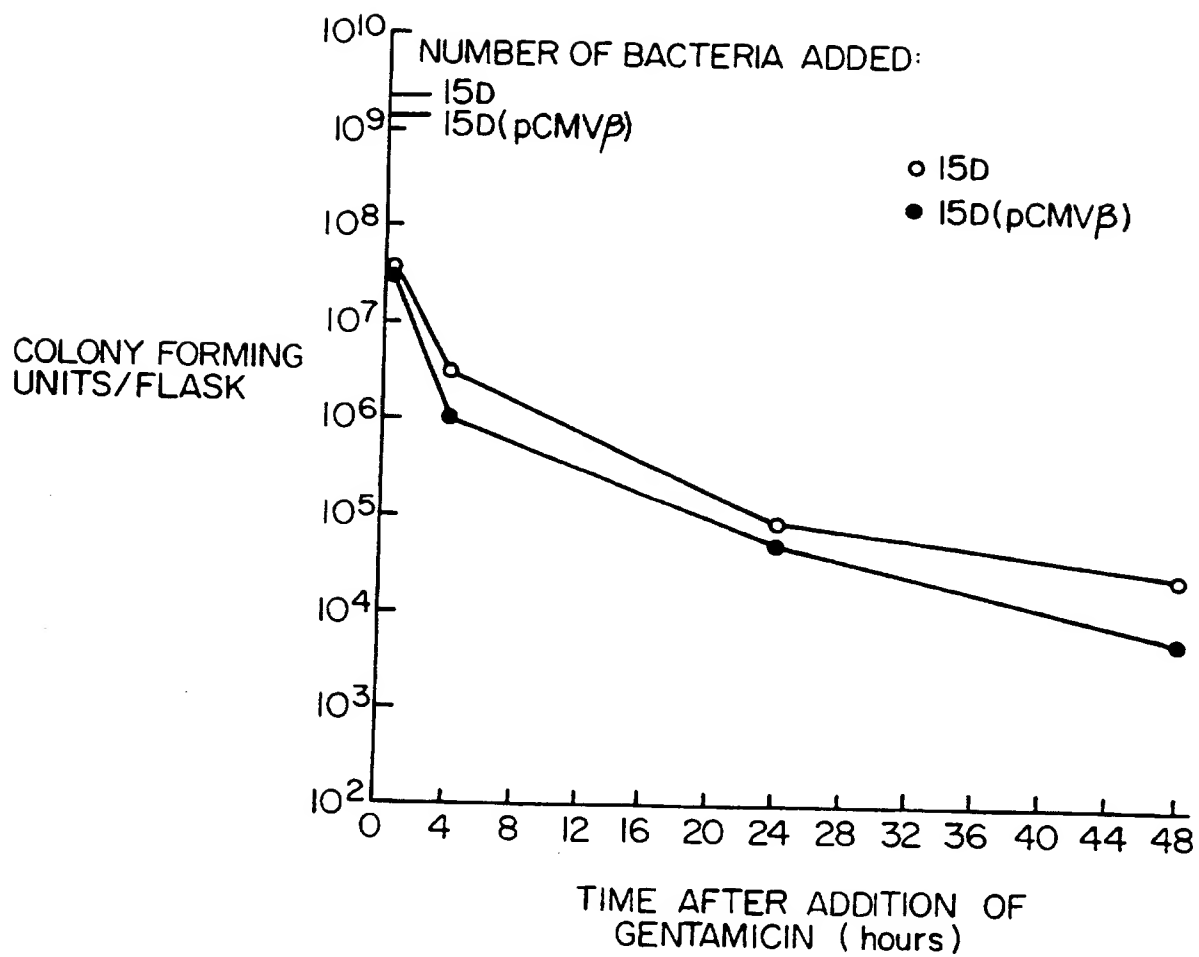
5 (ii) administering said bacteria to said cell.

FIG. 1



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FIG. 2A



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FIG. 2B

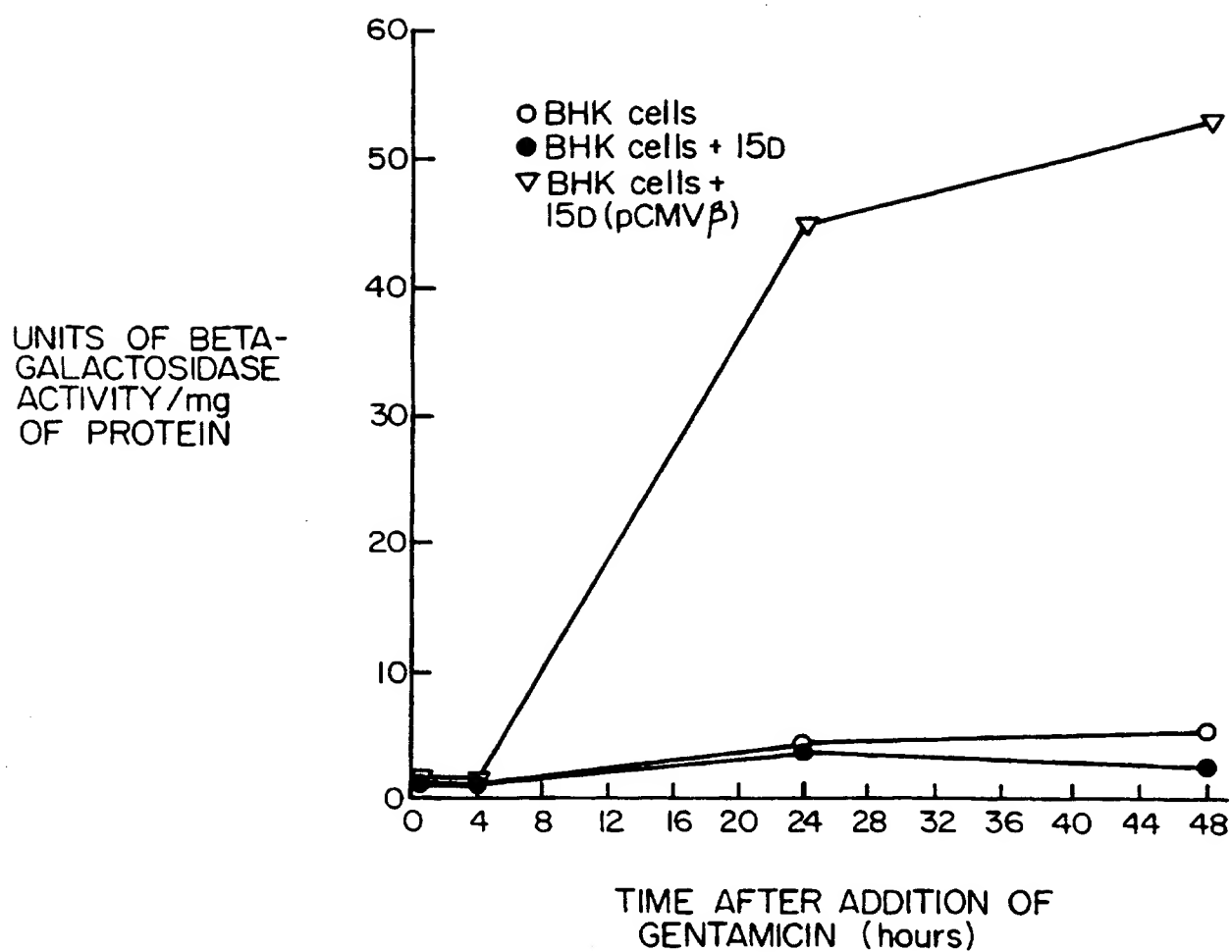


FIG. 3A



FIG. 3B

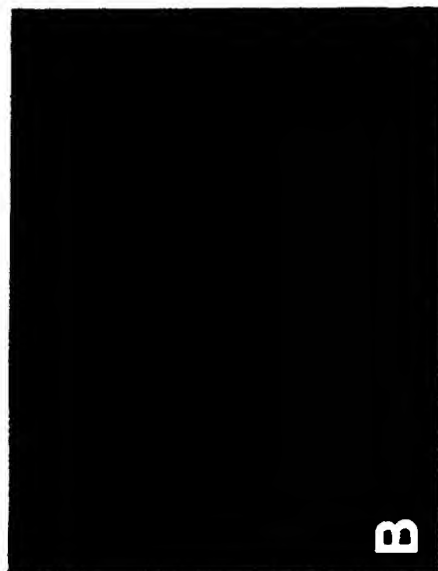


FIG. 3C

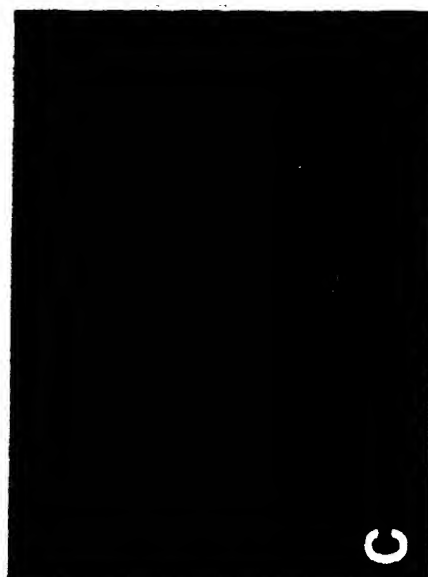


FIG. 3D

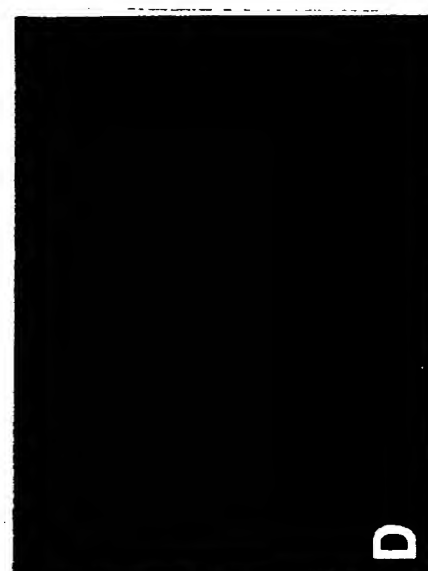


FIG. 3F

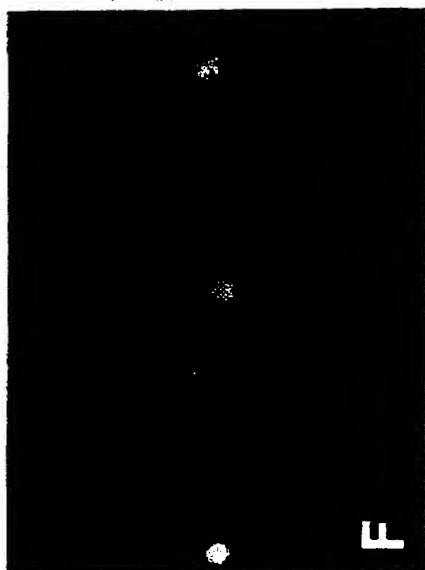


FIG. 3H



FIG. 3E

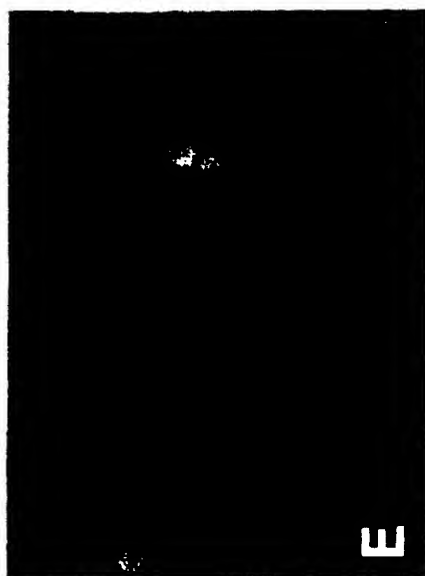


FIG. 3G

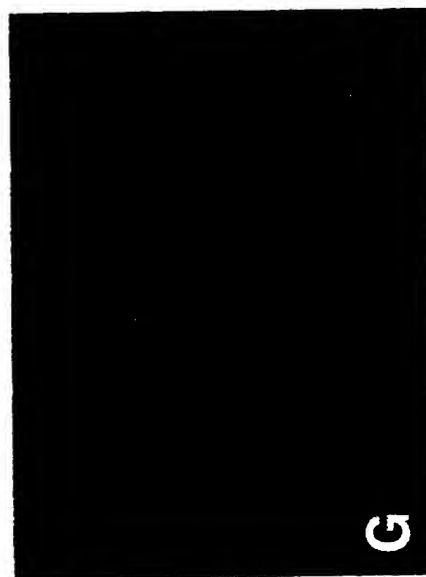


FIG. 4B

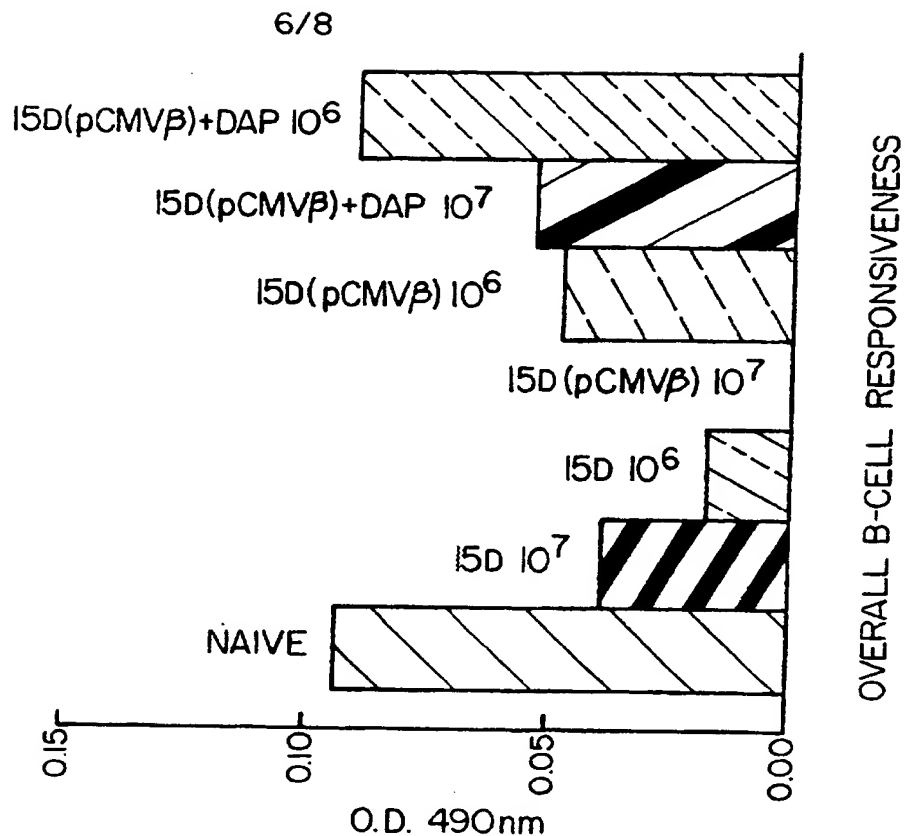


FIG. 4A

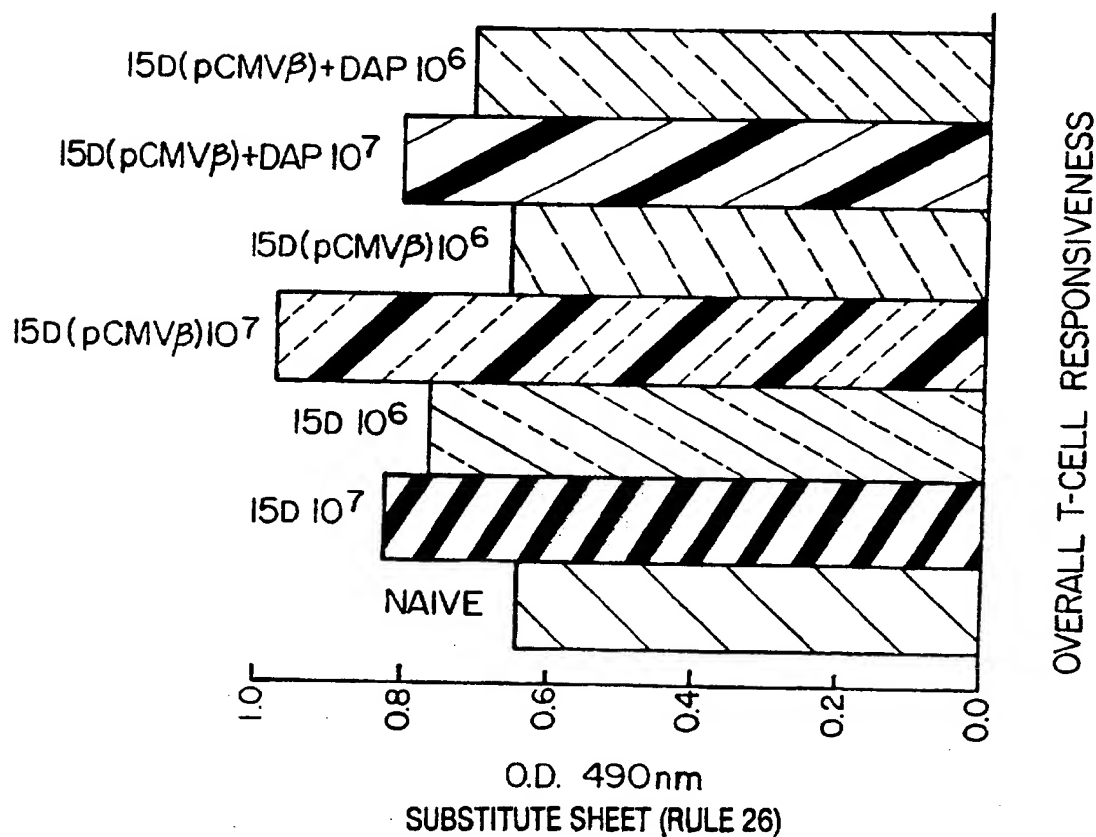


FIG. 4D

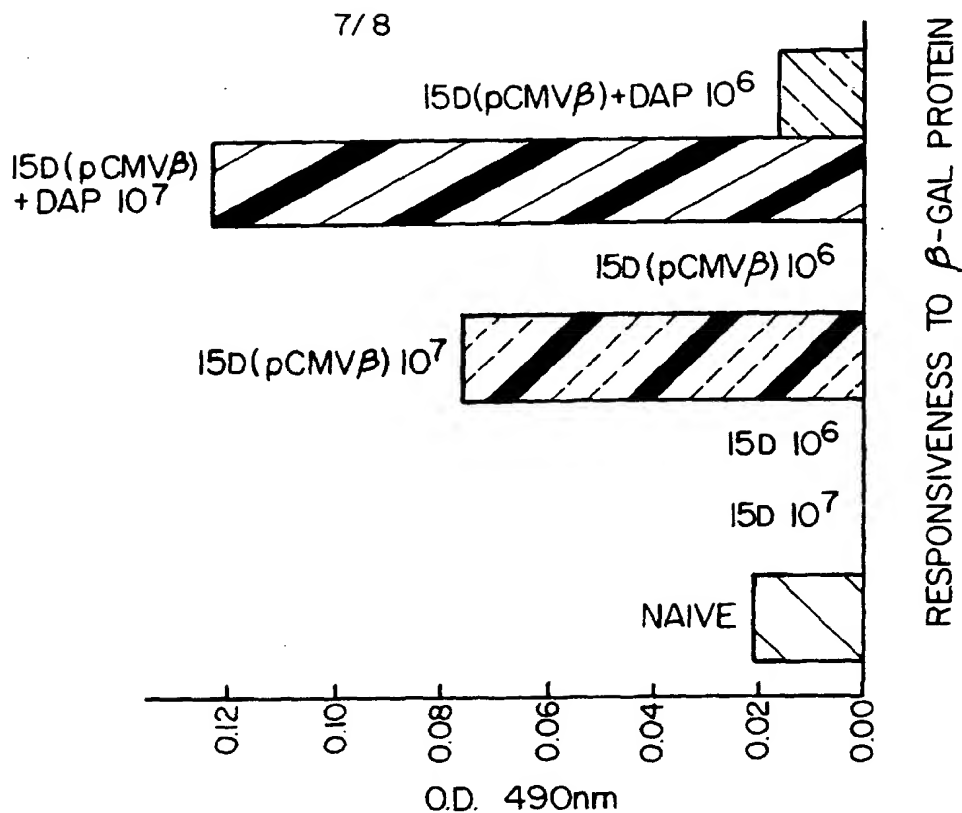


FIG. 4C

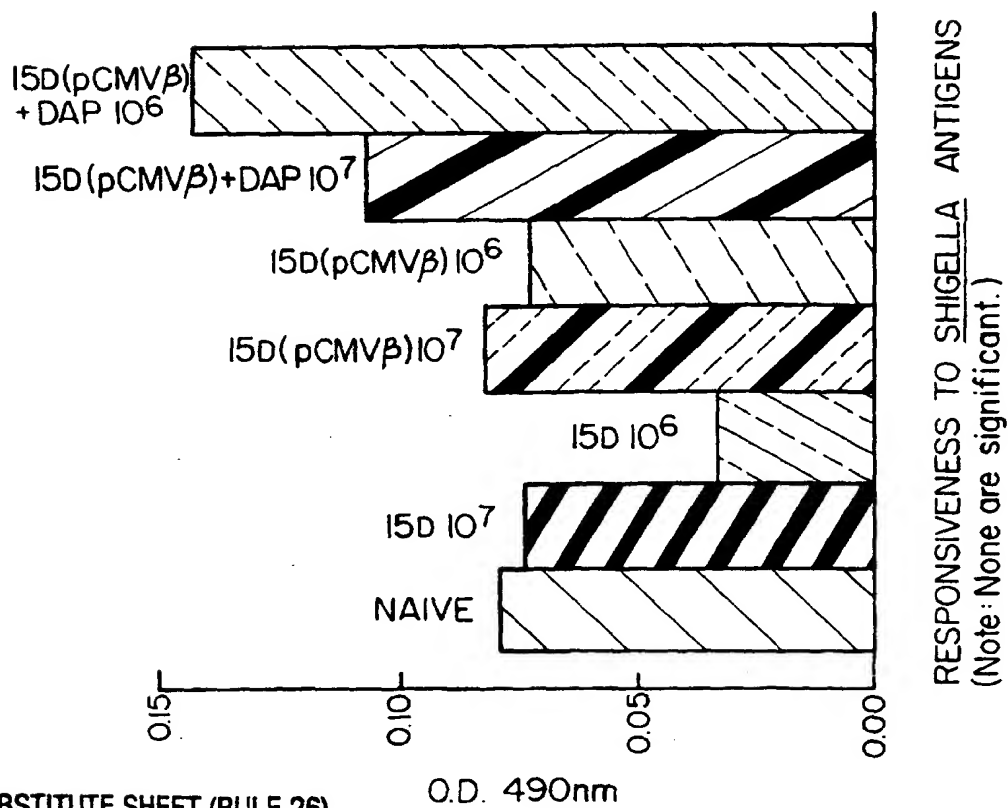
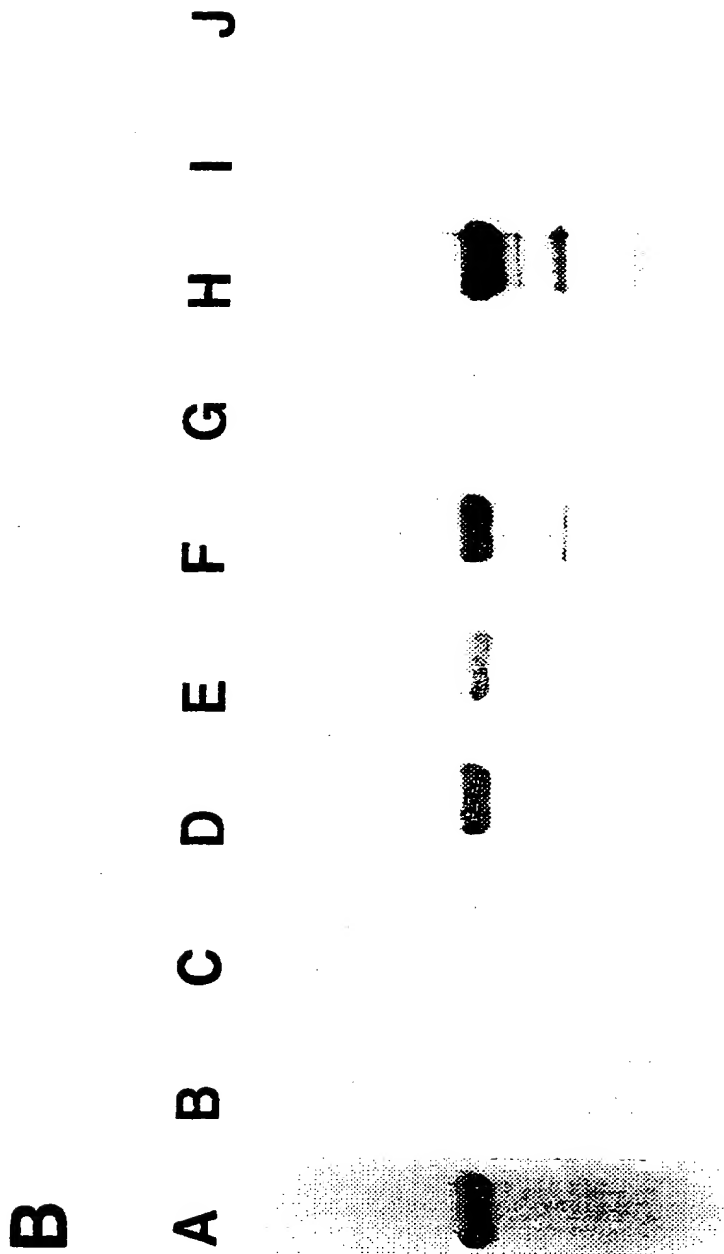


FIG. 5



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/14190

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 63/00; A61K 39/02; C12N 1/00, 1/36

US CL : 424/93.2, 234.1; 435/245,822

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2, 234.1; 435/245,822

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE

search terms: Shigella, attenuate#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Database Medline, US National Library of Medicine, (Bethesda, MD, USA), No. 88266201, LINDBERG et al. Development of an auxotrophic oral live Shigella flexneri vaccine. Vaccine. April 1988. Vol. 6, No. 2, see entire Abstract.	1, 2, 11-13 ----- 3-6
X -- Y	Database Medline, US National Library of Medicine, (Bethesda, MD, USA), No. 96012507, PHALIPON et al. Live attenuated Shigella flexneri mutants as vaccine candidates against shigellosis and vectors for antigen delivery. Biologicals. June 1995. Vol. 23, No. 2, see entire Abstract.	11-13 ----- 1-6
X -- Y	US 4,632,830 A (FORMAL ET AL.) 30 December 1986, see entire document.	11-13 ----- 1-6

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application best cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* documents which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

02 DECEMBER 1996

Date of mailing of the international search report

31 DEC 1996

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

CHRISTOPHER R. TATE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/14190

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
X — Y	US 5,077,044 A (STOCKER) 31 December 1991, see entire document	11-13 — 1-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/14190

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6 and 11-13

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/14190

BOX II OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Search Authority has found 10 inventions claimed in the International Application covered by the claims indicated below:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

- I. a. Claims 1-3 drawn to a first product, an attenuated *Shigella* strain.
b. Claims 4-6, drawn to a method of making the first product.
c. Claims 11-13, drawn to a first method of use of the first product, a method of reducing disease symptoms.
- II. Claims 7-10, drawn to a second product, a vaccine.
- III. Claims 14-19, drawn to a third product, a DNA delivering system.
- IV. Claims 20-23, drawn to a fourth product, an antigen delivery system.
- V. Claims 24-27, drawn to a method of oral immunization.
- VI. Claims 28-33, drawn to a method of delivering DNA to a cell.
- VII. Claims 34-39, drawn to a method of delivering an antigen to a cell.
- VIII. Claim 40, drawn to a method of detecting *Shigella* infection.
- IX. Claims 41-43, drawn to a fifth product, a diagnostic test kit.
- X. Claim 44, drawn to a method of delivering nonspecified functional nucleic acids to a cell.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I-X do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups I, II, III, IV, and IX are each drawn to distinct products as evidenced by the claims themselves: a strain of *Shigella*, a vaccine, a DNA delivery system, an antigen delivery system, and a diagnostic test kit. The inventions of Groups II, III, IV and IX do not require the attenuated *Shigella* strain of Group I because attenuation can be achieved by numerous means other than that of Group I, such as serial passaging or mutating a different *Shigella* gene to create an avirulent strain. Groups V, VI, VII, VIII, and X are each drawn to independent methods which do not share a special technical feature with any of the other groups.

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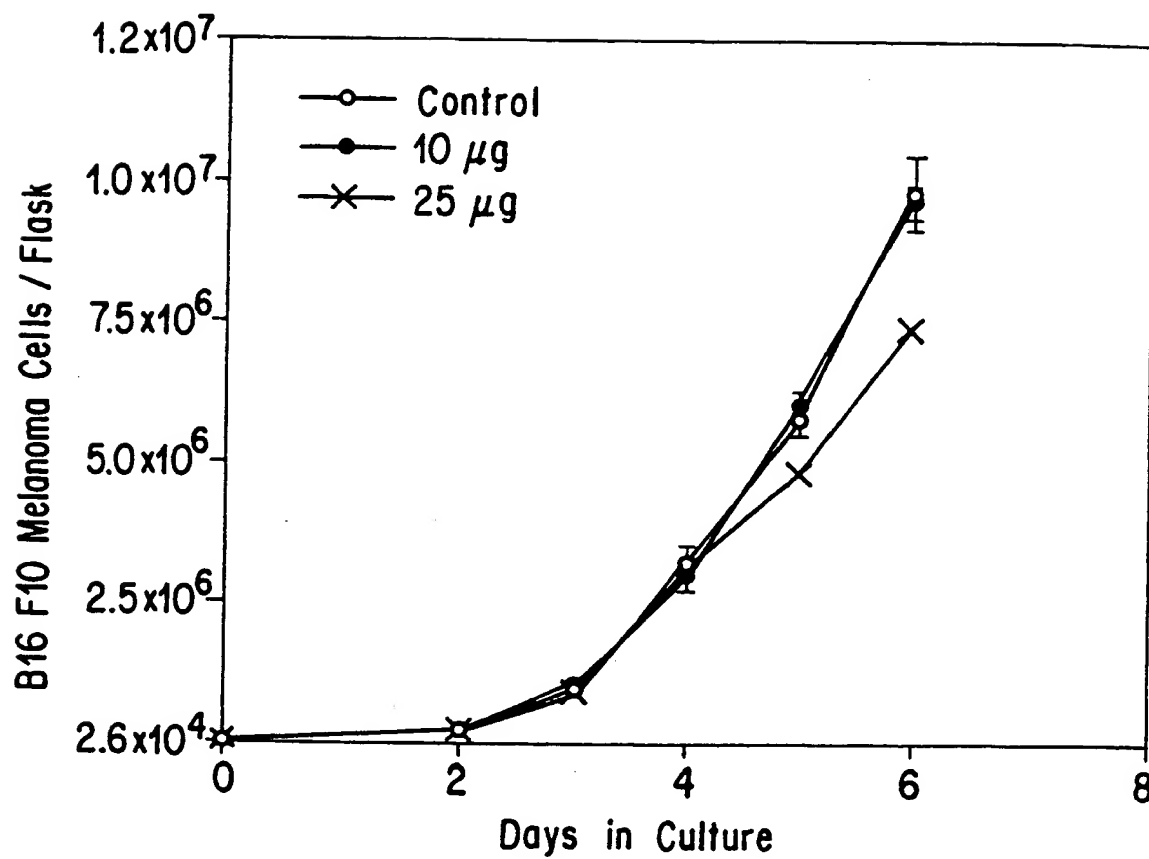


FIG. 11F

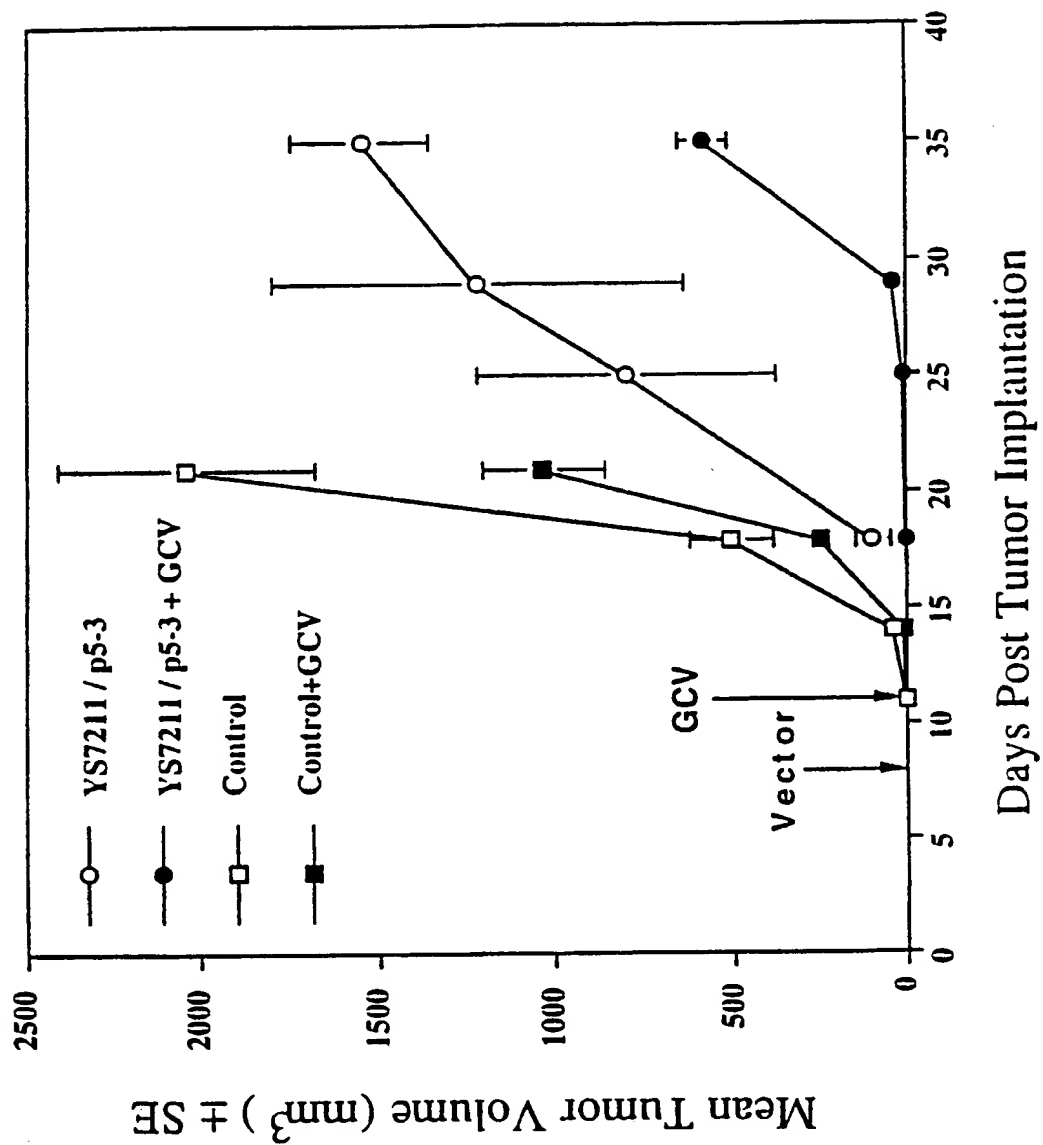


FIG.11G

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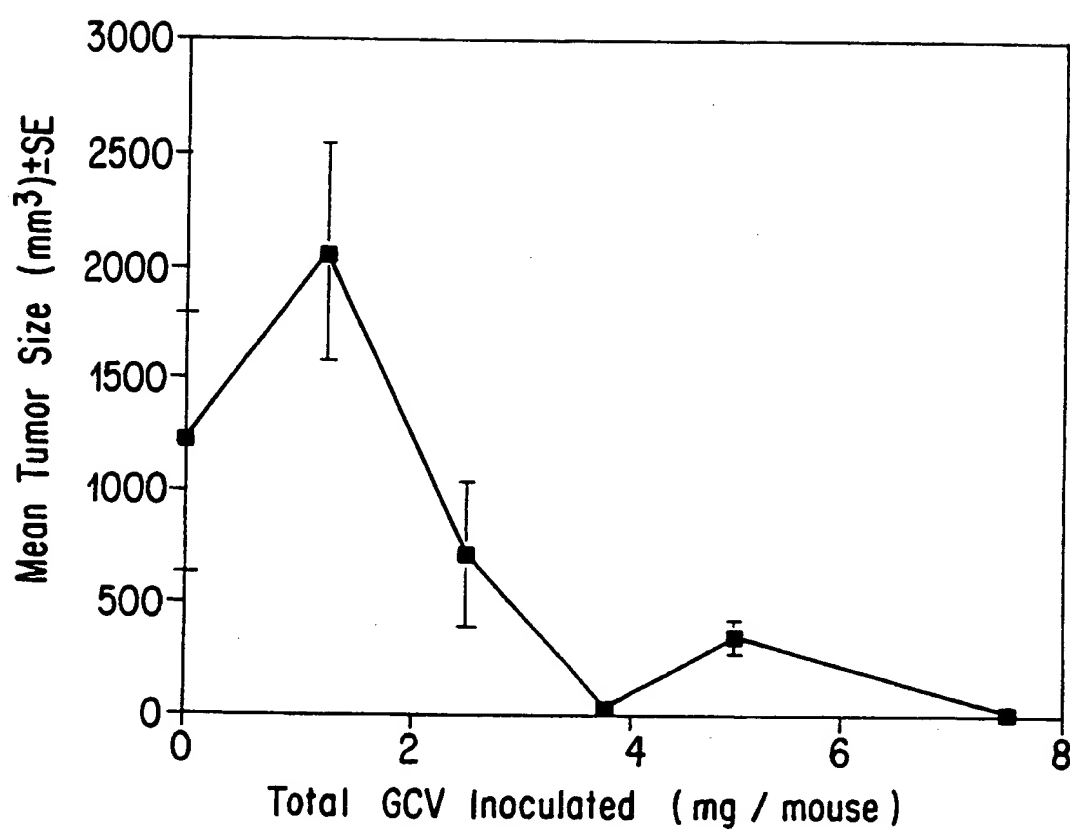


FIG. 11H

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FIG.12A

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FIG. 12B

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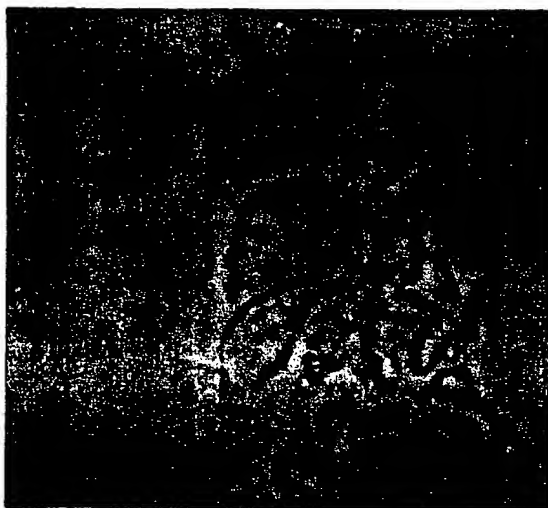


FIG.13A

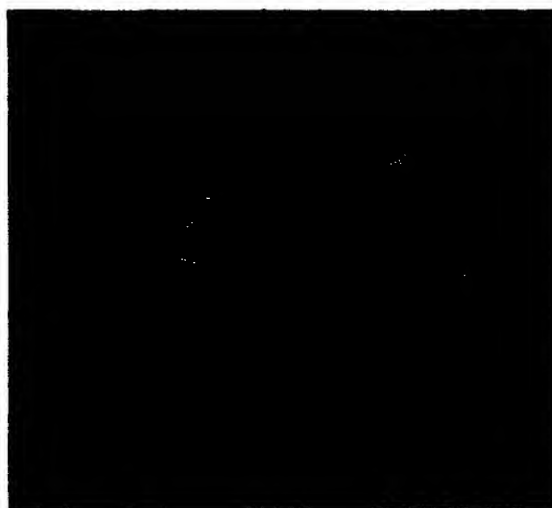


FIG.13B

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FIG.14A

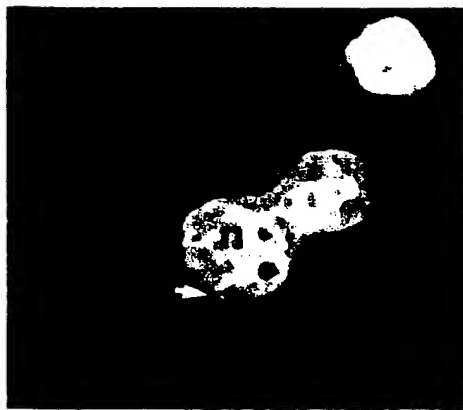


FIG.14B



FIG.14C

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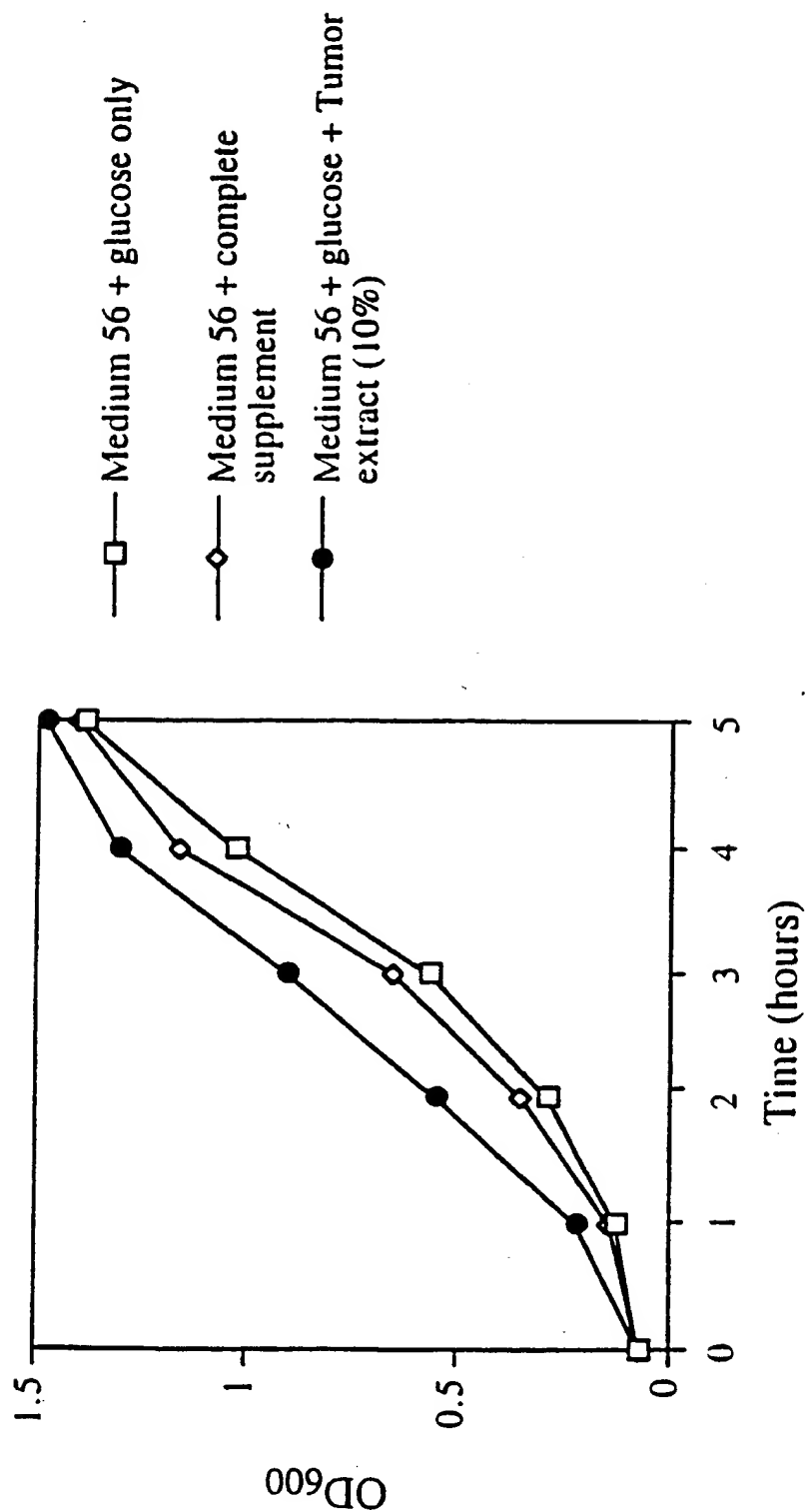


FIG. 15A

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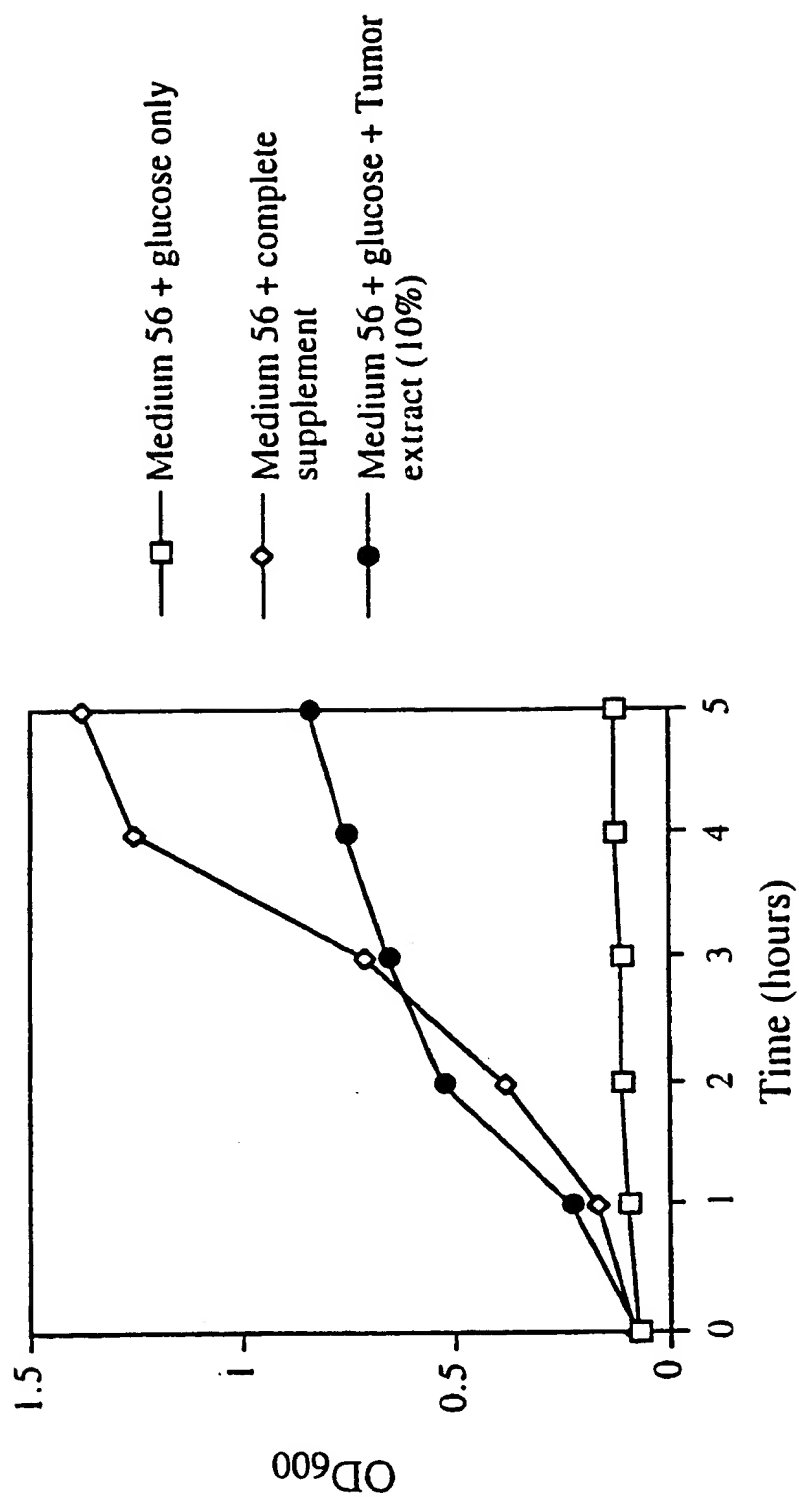


FIG.15B

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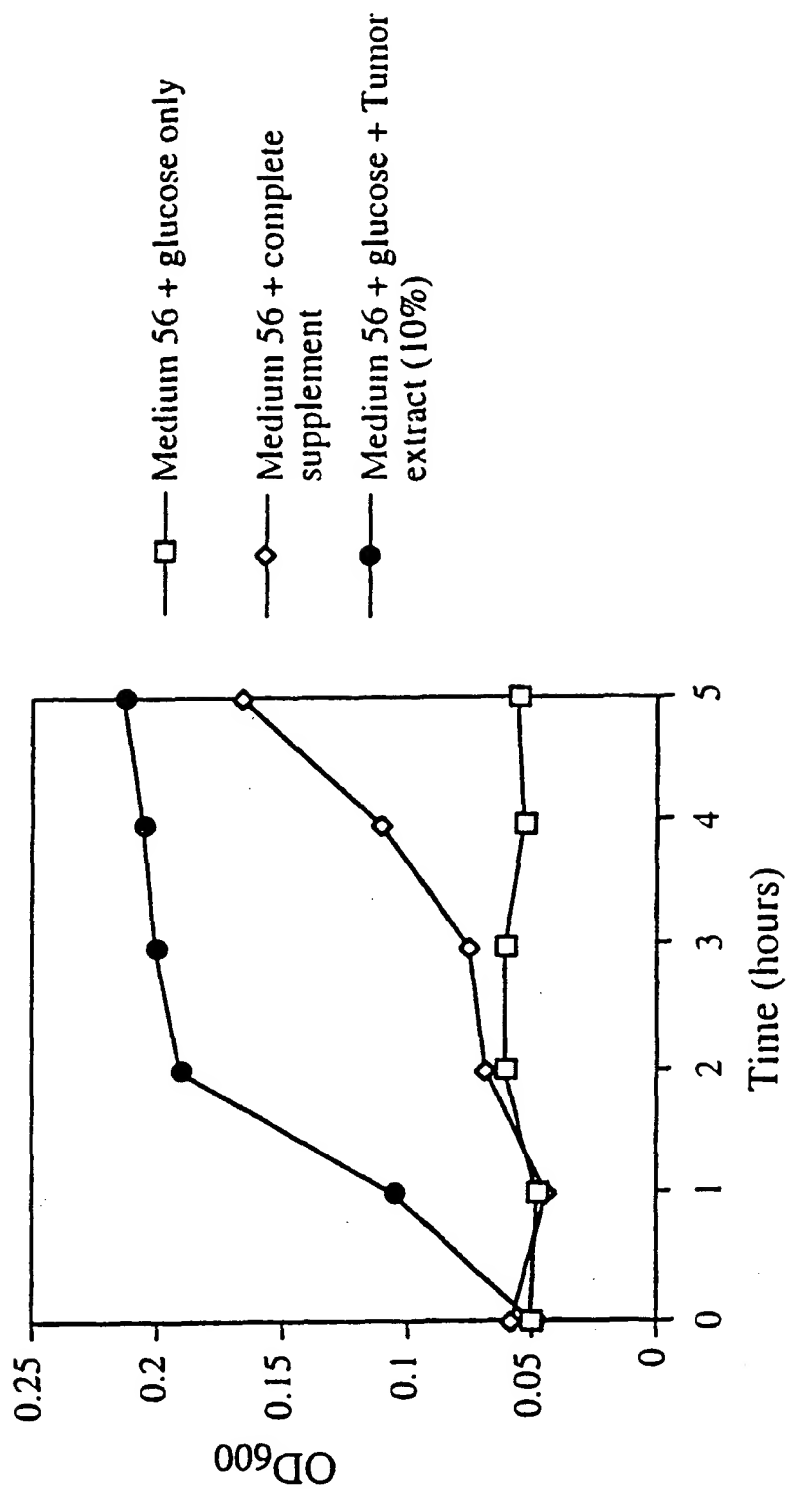


FIG. 15C

Growth of Clones 72 and YS7212 in Human M2 Melanoma Cultures

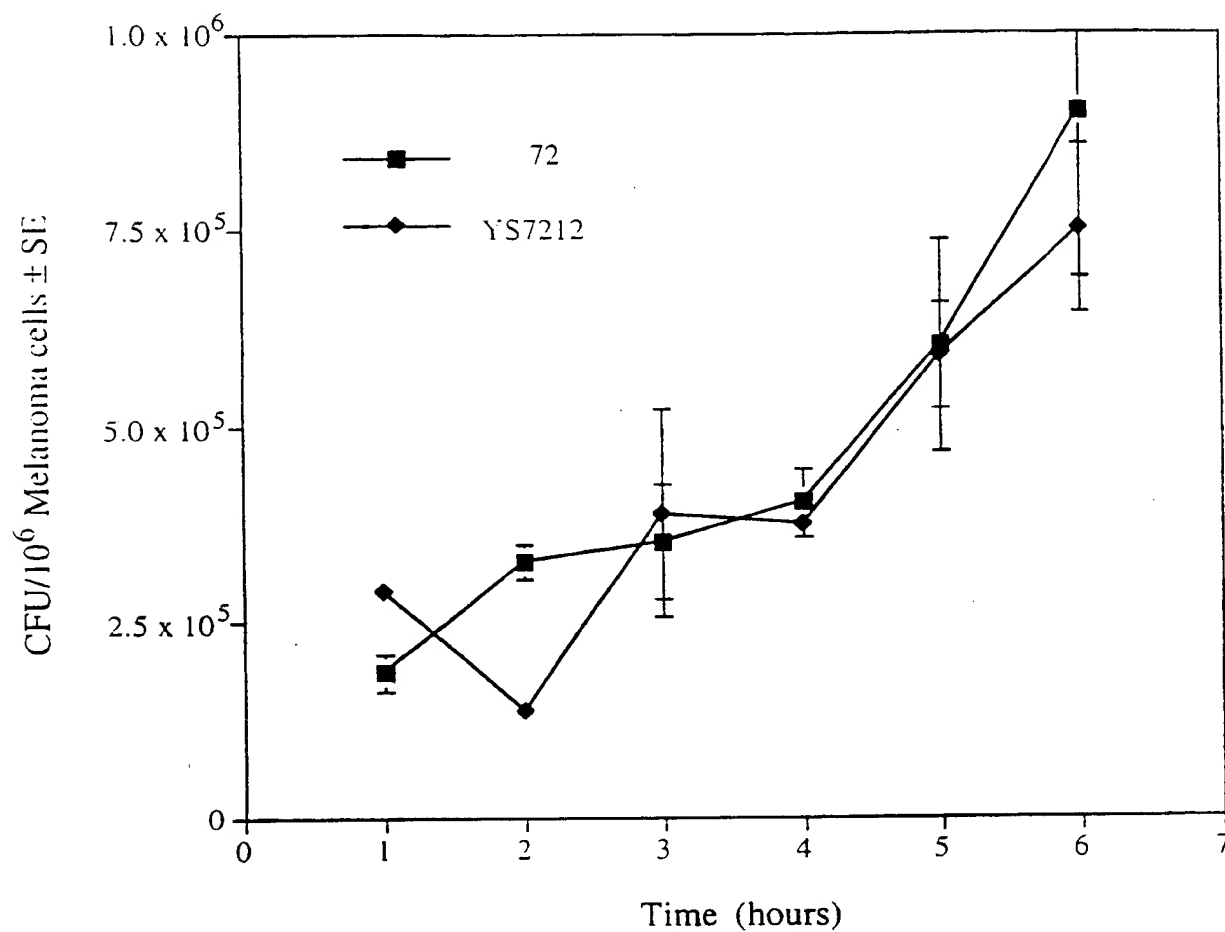


FIG. 15D

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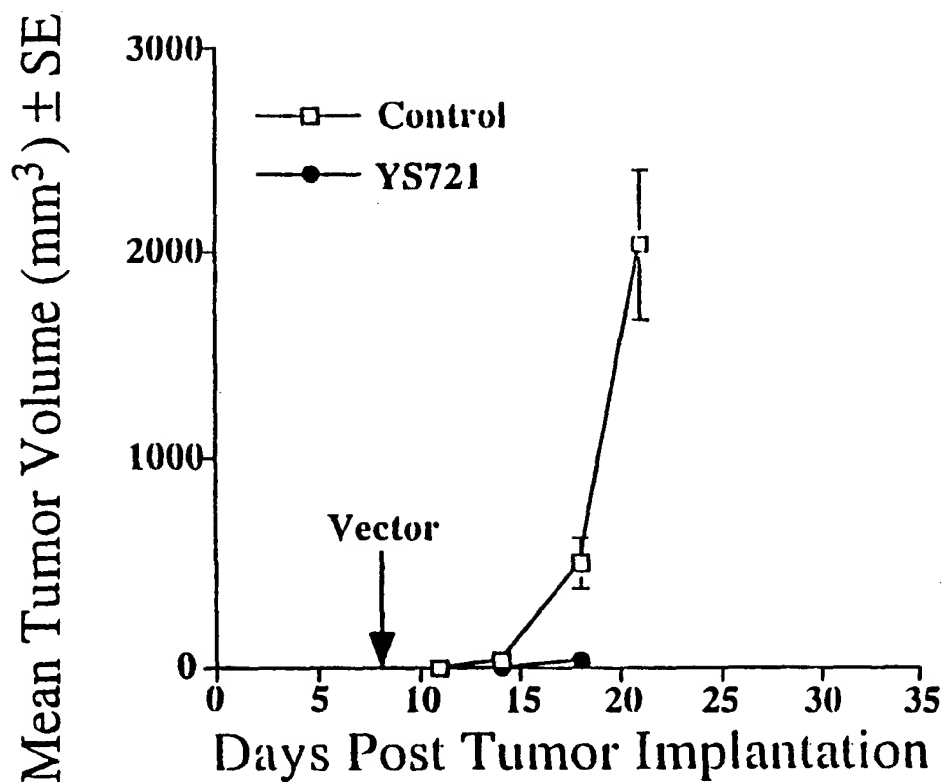


FIG. 16A

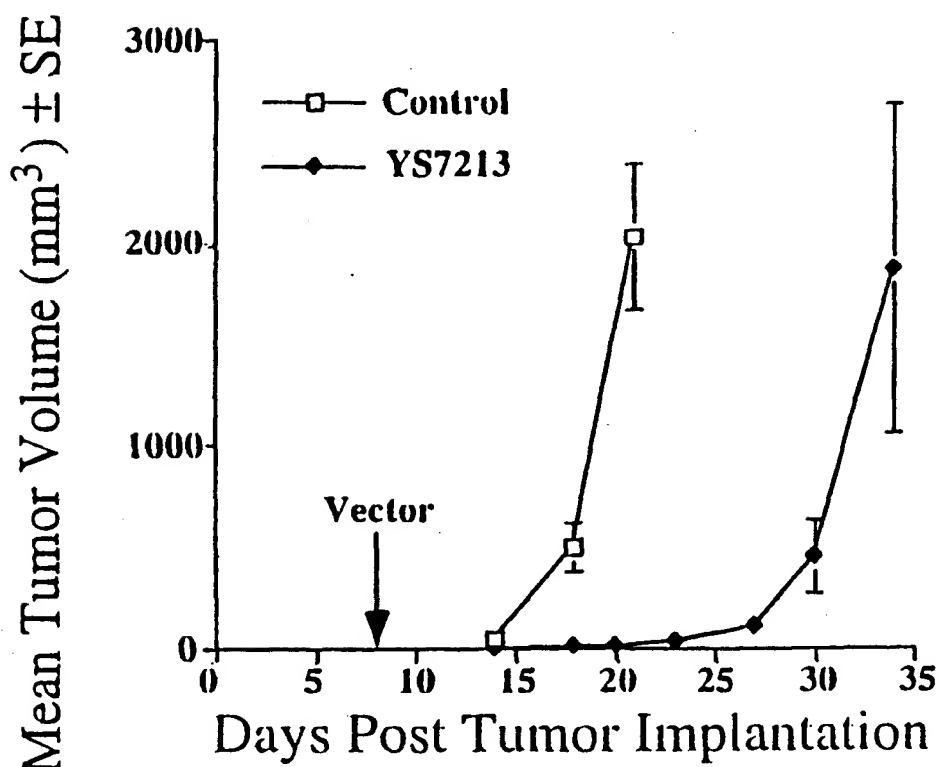


FIG. 16B

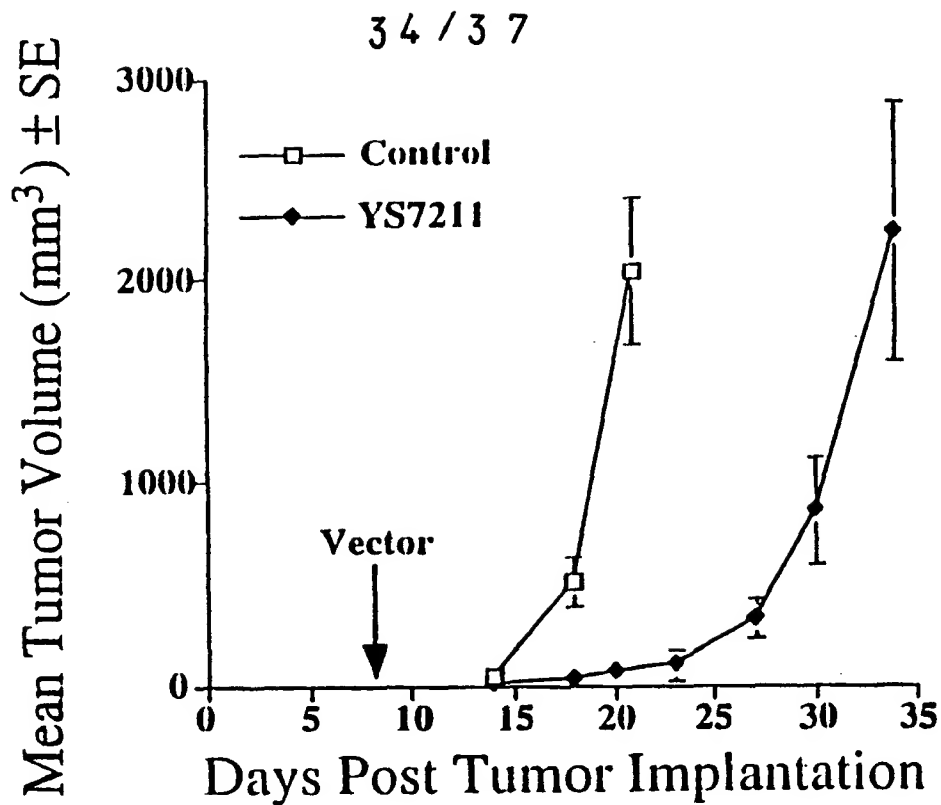


FIG. 16C

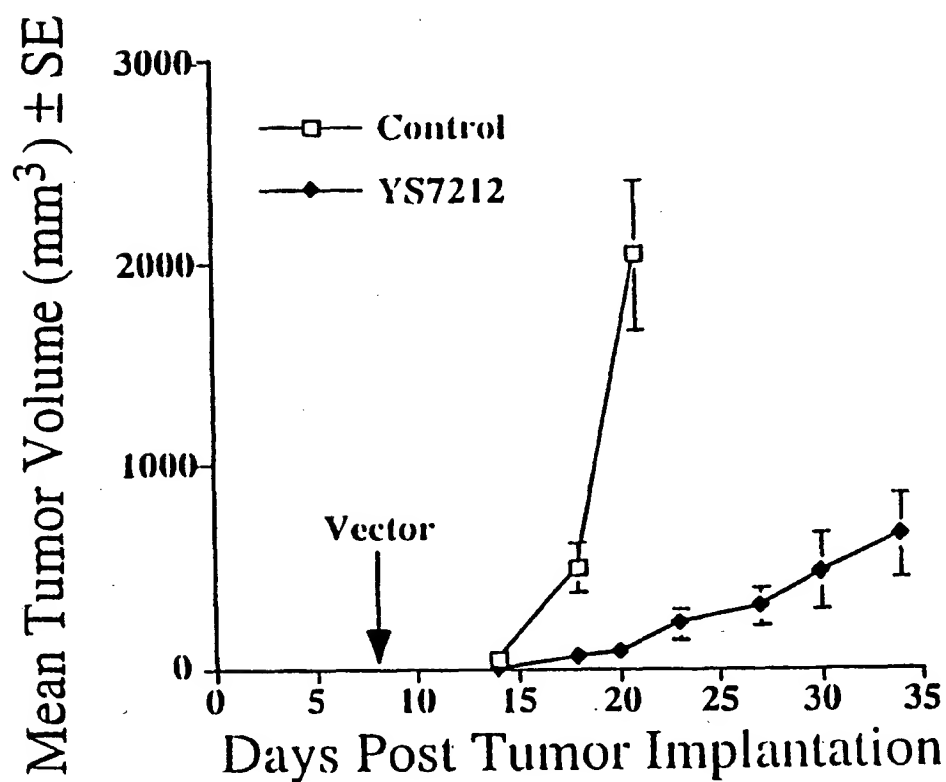


FIG. 16D

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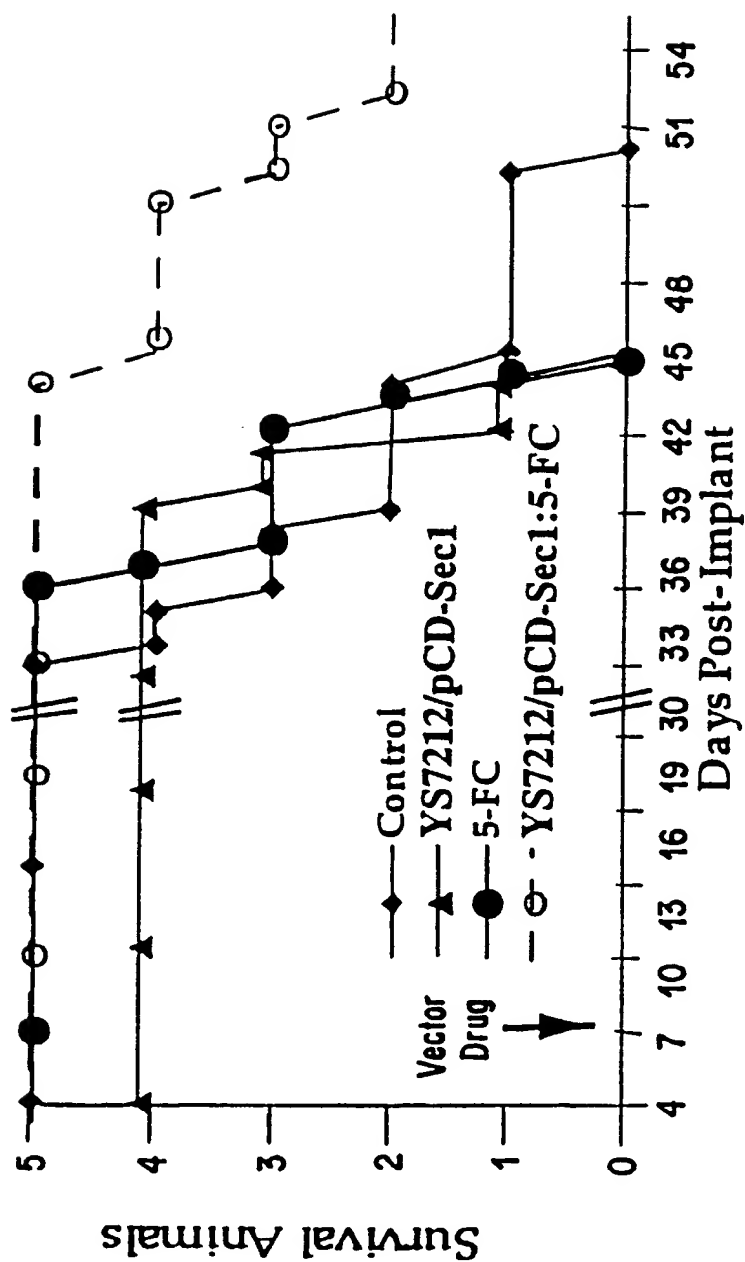


FIG.17

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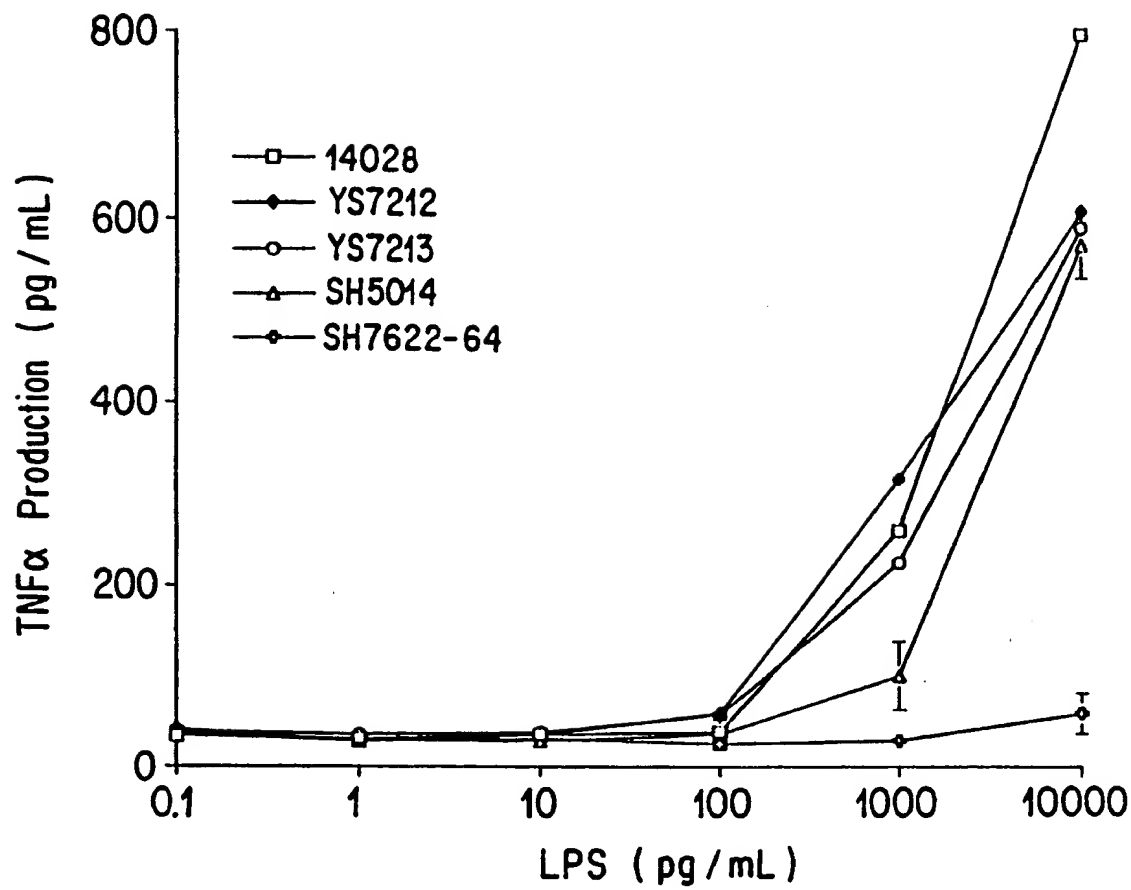


FIG. 18

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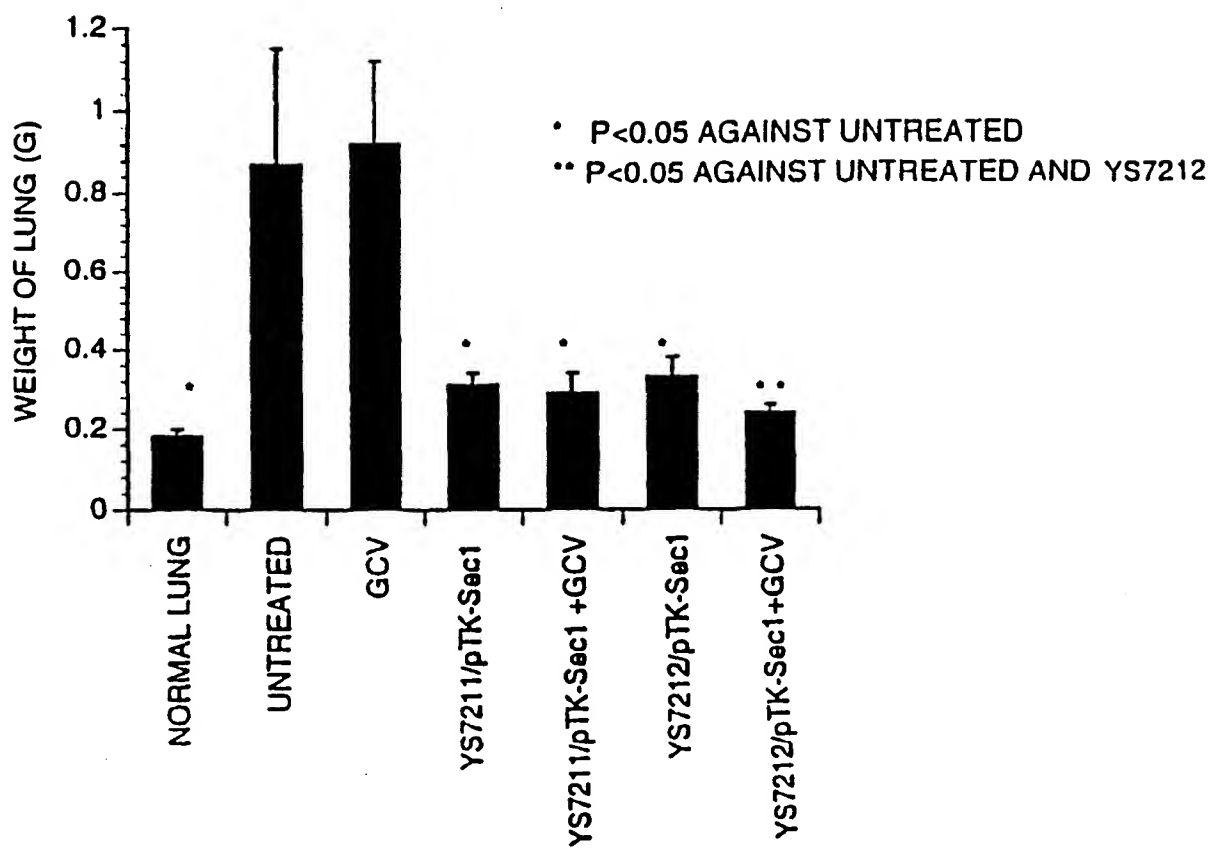


FIG. 19

INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US96/10250
A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/234.1, 258.1; 435/4, 6, 29, 252.1, 252.3, 252.8, 320.1, 879; 436/64; 530/351; 935/22, 27, 29, 72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, DERWENT

search terms: salmonella?, typhimurium?, cancer?, tumor?, tumour?, treat?, administ?, diagnos?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MCLAUGHLIN et al. Synergistic Activity of Components of Mycobacteria and Mutant Salmonella in Causing Regression of Line-10 Tumors in Guinea Pigs. Cancer Research. May 1979, Vol. 39, No. 5, pages 1766-1771, see entire document.	1-65
A	US 4,436,727 A (E. E. RIBI) 13 March 1984, see entire document.	1-65
A	US 5,021,234 A (U. EHRENFELD) 04 June 1991, see entire document.	1-65
A	US 5,344,762 A (A. KARAPETIAN) 06 September 1994, see entire document.	1-65

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

21 AUGUST 1996

Date of mailing of the international search report

10 SEP 1996

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

NANCY J. DEGEN

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10250

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10250

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 39/02, 39/112; C07K 14/525; C12N; 1/02, 15/63, 15/74; G01N 33/48

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/234.1, 258.1; 435/4, 6, 29, 252.1, 252.3, 252.8, 320.1, 879; 436/64; 530/351; 935/22, 27, 29, 72

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-4, partially, 5-43, and 57-65, partially, drawn to *Salmonella typhimurium* strains, plasmids carried by these strains, kits containing the bacteria and a method of treating cancer using the microorganisms.

Group II, claims 1-4, partially, 44-56, and 57-65, partially, drawn to *Salmonella typhimurium* strains, plasmids carried by these strains, kits containing the bacteria and a method of diagnosing cancer using the microorganisms.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Although the *Salmonella* strains are the same, they are used for two distinct purposes; the treatment of cancer and the diagnosis of cancer. These two methods are distinct because the ultimate goals are different and the steps used in carrying out these aims are also different. Note that PCT Rule 13 does not provide for multiple methods of use within a single application. (37 C. F. R. 1.475(d)).